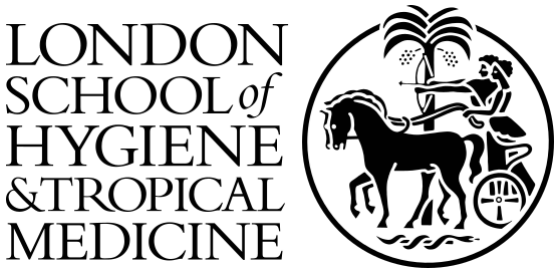

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# The Biology of Visceral Leishmaniasis Vectors in the San Andrés de Sotavento Focus, (Cordoba Colombia).

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Dissertation submitted to the
Faculty of Science

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**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Throughout its range of South and Central America, visceral leishmaniasis due to *Leishmania chagasi* is transmitted by *Lutzomyia longipalpis*. Recently, a new vector, *Lutzomyia evansi*, has been discovered transmitting the parasite in the Caribbean Coast of Colombia.

Field studies, using both experimental and observational methodologies were employed to elucidate the main ecological and behavioural factors affecting disease transmission in the focus of San Andrés de Sotavento, northern Colombia. Nine species of *Lutzomyia* were present and *Lu. evansi* constituted 90% of all sandflies caught. Flies were most abundant in April, May June and September. Trapping in and around houses showed *Lu. evansi* to be endophilic but with exophagic behaviour, preferring houses near to forest edge as resting places.

Host preference, measured using a newly designed trap in a rotational experimental design, showed that humans were preferred over dogs or opossums (reservoirs) during the peak abundance of *Lu. evansi*. This was supported by catches on tethered hosts and bloodmeal analysis although location of capture of resting flies was also a significant factor.

Mark-release-recapture studies showed that *Lu. evansi* can move up to 800m after 5 days and that freshly fed flies move a few hundred metres to resting sites.

Basic life history data on *Lu. evansi* was obtained from laboratory rearing. This species was bred under laboratory conditions though high mortalities were seen in first instars. In adults survival was associated with different types of sugar.

Flagellate parasites resembling *L. chagasi* were found in 3 of 5326 wild caught *Lu. evansi* (0.05%) however, culturing and subsequent characterization of these isolates failed. Experimental infections with *L. chagasi* showed that at least one strain of the parasite grew more prolifically in *Lu. longipalpis* than in *Lu. evansi*. This, together with a limited vector range compared to the Old World *L. infantum* is suggested to be the result of a recent parasite-vector association.

Morphologically no differences were seen between Colombian, Venezuelan and Costa Rican *Lu. evansi* populations. Some variation was seen however in one enzyme (6GPDH) of 18 isozymes tested. Mitochondrial DNA variation was seen between Central and South American populations.

## Acknowledgements

Now that this thesis has been finished and the work appears under my name, I would like to give credit to the people that in one way or another were involved with my project. Indeed it would be a difficult task to thank everyone, hence this represents a selection of the names of institutions and people who most contributed to the success of my study. Collaborations of a diverse manner were received from different people linked to me institutional, academic, colleagues and familiar ties.

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Finally, I would like to dedicate this work to my beloved family Luz Elena, Santiago and Jacobo.

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# CHAPTER 1

# INTRODUCTION

## 1.1 The leishmaniases: an overview

The leishmaniases are included among those diseases that deserve special public health attention. After malaria, onchocerciasis and Chagas disease, they constitute the fourth important group of insect borne parasitic diseases of humans. Leishmaniases are widely distributed in tropical and subtropical areas of Asia, Africa, the Mediterranean basin and the New World. According to WHO studies, approximately 350 million persons are at risk of contracting these diseases (Desjeux, 1992) and there is a global estimation of 600 thousand new cases/year and 12 million are infected at any one time. However, is thought that these global figures are underestimates.

### 1.1.1 Cycle of transmission of leishmaniases

The basic transmission dynamics involve the interaction of more than 20 species of trypanosomatid flagellate protozoa as parasites, over 70 phlebotomine sandfly species as vectors and some wild and domestic animals acting as reservoir-hosts of the disease.

1.1.1.1 The parasite: Despite their wide geographic range and different clinical manifestations, all leishmaniases are due to species of parasites belonging to the genus *Leishmania* Ross 1903. As defined by Lainson and Shaw (1987) this group includes digenetic Protozoa (Kinetoplastida: Trypanosomatidae) with flagellar forms (promastigotes and paramastigotes) in the alimentary tract of the insect host, and rounded forms (amastigotes) living and dividing in macrophages of a vertebrate host. None of the forms have yet been shown unambiguously to have sexual multiplication but all are known to divide by simple binary fission.

In the past, the classification of *Leishmania* species was largely based on their clinical manifestations supplemented with epidemiological and geographical distribution. Nowadays, with the advent of powerful tools, much light has been shed on the taxonomy of *Leishmania* based on biochemical, DNA and immunological techniques, complemented by characteristics of development in the vector and host. In 1987, Lainson & Shaw proposed taxonomic criteria

based on the development of species of *Leishmania* in the vector. According to these authors the parasites can be grouped into two subgenera: *Leishmania* (Saf'janova, 1982) and *Viannia* (Lainson & Shaw 1987). In its turn, the first subgenus is divided into three complexes, namely, *Leishmania* (*L.*) *donovani* complex, the *Leishmania* (*L*) *mexicana* complex and *Leishmania* (*L.*) *hertigi* complex. Both cutaneous and visceral disease are produced by these parasites. The subgenus *Viannia* consists of species in the *Leishmania* (*V.*) *braziliensis* complex, and an additional six unnamed species (Young & Arias, 1991). These parasites produce cutaneous and mucocutaneous disease. Although the Shaw and Lainson classification is widely accepted there is still some disagreements (see Grimaldi & Tesh, 1993; Grimaldi **et al.**, 1989; Añez **et al.**, 1989; Walters, 1993).

1.1.1.2 The vector: Around the world, the only proved transmitters of *Leishmania* parasites are blood-sucking sandflies (Diptera: Psychodidae: Phlebotominae). Over 700 species have been described and grouped in six genera, three of them (*Phlebotomus*, *Sergentomyia* and *Chinius*) restricted to the Old World and the remaining *(Lutzomyia*, *Warileya* and *Brumptomyia*) to the New World. However, only those belonging to *Phlebotomus* and *Lutzomyia* have importance in the transmission of disease. At present a total of 39 species of *Phlebotomus* and 88 of *Lutzomyia* (Killick-Kendrick, 1990; Young & Arias, 1991) are considered as suspected or proven vectors of *Leishmania*. However, it is thought that more vectors remain to be discovered.

In morphological terms, all species of Phlebotominae share a common pattern. Typically, both female and male sandflies are very small (ca. 5mm). Their heads present two compound eyes but lack ocelli, conspicuous maxillary palps and two antennae. In females, the mouthparts are modified to cut the skin of vertebrates (Lewis, 1975). The length and morphology of these appendages are important characters for systematic studies, especially for New World species (Young, 1979). In both sexes, the wings are unspotted, pointed apically and heavily covered with hair-like scales. Their legs are long, slender and have short spines on the hind femurs. The thorax is infuscated and less conspicuously hairy than the wings. The abdomen is oval in shape and its terminal segments are important for gender differentiation. Thus, males have conspicuous external, bilateral and symmetrical genitalia, which rotate 180° after eclosion. Conversely, females lack these structures but have internal paired spermathecae, important for taxonomic identification of species. An additional pattern in the classification of Old World species is the setation of abdominal tergites II to VI.

However, this pattern has little importance for the New World species.

Despite the structural similarity of phlebotominae flies, each species (or group of them) displays different bio-ecological attributes. For instance, the information gained on ecological and behavioural aspects of *P. papatasi* might be typical of other *Phlebotomus* flies but is unlikely to be useful for extrapolating to *Lutzomyia* species.

1.1.1.3 Reservoir hosts: Despite the importance of vertebrates as hosts for *Leishmania*, the criteria for incriminating animal species as reservoirs has been the subject of much controversy. Most problems come from misuse of the terms host and reservoir. In epidemiological terms, a reservoir of *Leishmania* is an animal in which the parasite population is maintained indefinitely. The reservoir might or might not present symptoms. Conversely, a host is considered as an animal in which the parasite can exist and develop. There are situations where the main reservoir and final host of infection are the same (eg. Humans are the host and reservoir in the epidemic foci of visceral leishmaniasis in India, Sudan and parts of Kenya). Rodents, edentates, canids and marsupials are the most common reservoirs (Lainson & Shaw, 1979). So far, there are no reports of leishmaniae in birds and amphibians, though they are fed on by sandflies.

1.1.2 Epidemiology

The terms anthroponoses and zoonoses have been coined to distinguish different transmission cycles. According to Lysenko & Beljaev (1987), in zoonotic forms of leishmaniases the role of humans in transmission is almost insignificant. Similarly, mammals other than humans have a slight or negligible contribution to maintaining the disease in anthroponotic foci. From this point of view, most leishmaniases are primarily zoonoses (Chang et al., 1985) rather than anthroponoses. Regardless of this, and from the epidemiological standpoint, the dynamics of any form of leishmaniasis depends on population fluctuations in any of its integral components (ie. parasite- vector(s)- reservoir(s)). The American leishmaniases present greater diversity in parasite, vector and reservoir species compared to those from the Old World. Also, due to ecological differences between the two continents, there are variations in the human-vector contact. The American forms are rural and often associated with forestry or agricultural activities (the main exception being visceral leishmaniasis) conversely many Old World leishmaniases are urban.

Globally, species of the *L. braziliensis* and *L. major* complexes are associated with the

highest morbidity rates (Ashford et al., 1992); however, the visceral forms due to the *L. donovani* complex are considered the most serious public health problems since the highest mortality are associated with them.

### 1.1.3. The *Leishmania donovani* complex

This is distributed worldwide producing a form of the disease referred to as visceral leishmaniasis (VL). The complex consists of *L. (Leishmania) donovani* (India, Africa); *L. (Leishmania) infantum* (Mediterranean, Asia, Near East) and *L. (Leishmania) chagasi* (Central and South America). These parasites usually replicate well in macrophages within tissues throughout the host body producing enlargement and marked alterations in function of the liver, spleen, bone marrow and lymph nodes (Marsden & Jones, 1985). The patient presents with progressive weakness, weight loss, low grade fever, anaemia, hepatosplenomegaly, subcutaneous oedema, ascites and bleeding disorders. Without adequate treatment this disease is fatal. A brief review of the geographical distribution, vectors and reservoirs for each VL parasite is given below.

#### 1.1.3.1 *L. donovani*

Strains of this parasite are present in both zoonotic and anthroponotic foci. The zoonotic form is found in the Afrotropical region (Sudan savanna, from Senegal to the Ethiopian border and south to central Kenya) and through the Palaearctic Region (from Portugal to China). On the other hand, the human-sandfly-human cycle has been demonstrated in India and is also suspected to occur in Kenya.

Although dogs have been found infected in Senegal it is still not clear which are the main reservoirs of the parasite. In Sudan, the rodent *Arvicanthis* has been found infected with *L. donovani* parasites and in Kenya, there is some evidence wild canids and dogs are reservoirs of the parasite. However, human movements or migrations might have played an important role in spreading the disease. In the Indian-Bangladesh-Nepal foci no wild reservoirs of the parasites are known yet, though domestic animals serve as a maintenance source of blood for the sandfly vectors. In spite of the fact that *P. papatasi* is by far the most common anthropophilic species in some *L. donovani* areas, it is extremely unlikely to be involved in the transmission of the parasite as has been suggested. Species such as *P. alexandri* in China, *P. martini* in Kenya and Ethiopia and *P. argentipes* in India have decisive role in the transmission in these areas.

1.1.3.2 *L. infantum*

In the Old World, VL produced by *L. infantum* extends as a zoonosis through the Mediterranean basin, Middle Asia and northern Africa. In the Mediterranean region, VL disease shares many characteristics (biological and geological) of the cutaneous foci due to *L. tropica*, and in some places the two forms may coexist (Ashford & Bettini, 1987) though *L. tropica* is probably anthroponotic in most places. An invariable characteristic of *L. infantum* VL is that the dog is the major domestic reservoir of infection, though in some areas rodents (eg. *Rattus* sp.) and wild canids (eg. red fox, jackal) are also involved and might contribute to the dissemination of parasites. In Mediterranean countries, visceral leishmaniasis due to *L. infantum* is reported more and more frequently in association with human immunodeficiency virus (HIV) cases (Pratlong et al., 1995).

The list of proven vectors of *L. infantum* is short. Experimental transmission and natural infection studies shown that *P. langeroni, P. perniciosus, P. chinensis, P. perfiliewi* and *P. ariasi* (Killick-Kendrick, 1990; Lane, 1993) are the main vectors in the Mediterranean region and some foci in Asia. Additional data are needed to decide whether or not *P. longiductus, P. neglectus* and *P. hindustanicus* are vectors of the parasite (Killick- Kendrick, 1990; Munir, 1994; Lane, 1993).

1.1.3.3 *L. chagasi*

According to Lainson & Shaw (1987), *Leishmania chagasi* is a native American parasite which is the etiological agent for American visceral leishmaniasis (AVL). In contrast, other authors consider this parasite as a subspecies of the Old World *L. infantum* (Killick-Kendrick et al., 1980; Killick-Kendrick, 1990 and references therein). Both theories have pros and cons; in this text we will accept the second theory.

*Leishmania chagasi* is widely distributed in 14 countries from Mexico to Argentina covering arid and semi-arid tropical zoogeographic areas (Morrison, 1994; Grimaldi and Tesh, 1993; Grimaldi et al., 1989; Killick-Kendrick, 1990; WHO, 1990). Historically, the distribution of *L. chagasi* has fitted well with the ecological habitats of *Lutzomyia longipalpis*. As a direct consequence of the interaction between the sandfly and the parasite in these areas, two basic AVL transmission patterns have been defined (Shaw & Lainson, 1987). The first is the classic hyperendemic AVL foci of the open, dry areas of northern Brazil (Ceará, Bahía, Piauí). The domestic dog or the wild fox *(Cerdocyon thous)* act as reservoir(s). The high incidence of human cases is correlated with the high natural infection rate of *Lu. longipalpis*,

clearly illustrating the role of this sandfly in AVL transmission (Lainson et al., 1985). The second pattern encompasses foci in semi-arid forest. Dogs are suspected to be the main domestic host-reservoirs with foxes and highly adaptable opossums likely to be the wild reservoirs. In contrast to the first pattern, occasional human cases are recorded and foci range from epidemic to hypoendemic (eg. Brazil: Marajó focus; Bolivia: Yungas focus; Colombia: El Callejón focus; Honduras: Cerro Grande; Mexico; Nicaragua; etc.). The role of *Lu. longipalpis* in these cases is always not obvious, and the incrimination of this sandfly as the vector is largely based on the coincidence of its distribution with that of AVL cases (Jeronimo et al., 1994; Velasco, 1973; Lainson et al., 1983; Le Pont & Desjeux, 1985) or sometimes on experimental infections (Lainson et al., 1977; 1985). Nevertheless, there are regions where AVL is present but *Lu. longipalpis* has never been detected and therefore a third AVL pattern should be defined comprising those foci with rare but autochthonous AVL cases where an alternate vector is likely (cf. Travi et al., 1990; Cat et al., 1974; Ayala et al., 1980; Iversson et al., 1979).

The possibility of the existence of an alternate AVL vector is not recent but, was suggested in 1964 by Pifano and Romero (1964; 1973) in Venezuela. They found VL transmission in Sucre State and Margarita Island in the absence of *Lu. longipalpis* and postulated *Lu. evansi* as the most likely vector. Similarly, other species of sandflies have been suspected as alternate AVL vectors (eg. *Lu. atroclavata* in Guadeloupe (Courmes et al., 1966); *Lu. antunesi* in Brazil (Ryan et al., 1984). However, there is insufficient evidence to incriminate these flies as AVL vectors. Recently, the case for the involvement of *Lu. evansi* in AVL transmission has been reopened. In Costa Rica, Zeledón et al., (1984) and Zeledón et al., (1989) investigating an atypical cutaneous leishmaniasis outbreak due to *L. chagasi* (where the vector remains unidentified) suggested alternation between *Lu. longipalpis* and *Lu. evansi* during the rainy and dry season. Substantial evidence was presented by Travi et al. (1990) working in a focus of AVL in northern Colombia, where *Lu. longipalpis* is totally absent, they found *Lu. evansi* naturally infected with *L. chagasi*. More recently, additional evidence has been collected in two Venezuelan foci of AVL. In the first, situated in Carabobo State, both *Lu. longipalpis* and *Lu. evansi* are sympatric but only the latter species was observed harbouring promastigote forms, not yet identified (D. Feliciangeli, pers. comm.). The second focus, occurs in Trujillo State, where AVL is in the absence of *Lu. longipalpis* and the main man-biter is *Lu. evansi* (Moreno & Oviedo, 1995).

## 1.2 Leishmaniases in Colombia.

### 1.2.1 Overview

Colombia is a country extremely rich in fauna and flora, which derives from its privileged geographical position in the tropics as well as from the structural complexity that the Andes mountains generate. Several climatic life zones have been delimited ranging from tropical to nival (with permanent snow) and from rain forest to desert brush (Espinal & Montenegro, 1963).

In Colombia, species of *Leishmania* and their vectors are literally distributed over all the country, and the leishmaniases are one of the main health problems due to parasites (Werner & Barreto 1981; Corredor et al., 1980). However, epidemiological reports of leishmaniases appear to reflect the human population densities and the availability of medical facilities. As a result, the distribution, morbidity and mortality of the different forms of leishmaniases are usually underestimated. For instance, in an analysis of the data recorded in the literature between 1872 and 1980, Werner and Barreto (1981) found from a total of 1,865 reported cases of leishmaniasis, AVL represented a tiny fraction (1.1%). However, after one year of data collection (1981) by the Colombian Ministry of Health, an increase of 250% over the number of all previous AVL cases was reported (Corredor et al., 1990).

The most recent figures available indicate a national increase in the annual incidence of all forms of leishmaniasis. Between 1981 and 1986 a total of 9,369 new cases of CL were reported (Corredor et al., 1990). This might in part be a reflection of the improvement in diagnosis as well as increased contact with *Leishmania* parasites and sandflies as result of the human colonization and military activities in forested areas (Montoya et al., 1990).

Four species of the *Leishmania* subgenus *Viannia* (*L. braziliensis, L. panamensis, L. guyanensis* and *L. colombiensis*) and three of the subgenus *Leishmania* (*L. chagasi, L. mexicana* and *L. amazonensis*) cause leishmaniases in Colombia (Grimaldi et al., 1989). The highest number of cases are found in the Pacific lowlands and the central part of the country, these and are cutaneous and mucocutaneous disease mainly caused by *L. braziliensis* and *L. panamensis*. *L. guyanensis* is almost completely restricted to the Amazon basin -Figure 1.1. (Travi et al., 1988; Corredor et al., 1986; Saravia et al., 1985; Werner and Barreto, 1981) while the recently named *L. colombiensis* occurs near the Venezuelan border (Kreutzer et al., 1991). On the other hand, the visceral form of the disease, due to *L. chagasi*, is endemic

through the Magdalena River Valley and dry areas of the Caribbean coast -Figure 1.2- (Blanco-Tuirán et al., 1993; Travi et al., 1990; Corredor et al., 1989a; Camacho-Sánchez, 1978). The cutaneous and diffuse forms due to *L. mexicana* and *L. amazonensis*, occur rarely in Santander, Nariño and Meta departments -Figure 1.1. (Rodríguez et al., 1985; Montoya et al., in prep.).

A total of nine *Lutzomyia* species, grouped in three subgenera, are regarded as proven or suspected vectors of *Leishmania* parasites in Colombia. A list of the most probable vectors, based either on findings of natural infections or on experimental or circumstantial evidence, is given in Table 1.1

As far as reservoirs are concerned, many species of mammals (especially rodents) have been studied in the search for potential sources of parasites. However, the list is limited to two confirmed cases: the sloth *Choloepus hoffmani* which harbours *L. panamensis* (Loyola et al., 1988a) and the domestic dog incriminated as a reservoir of *L. chagasi* (Corredor et al., 1971). The opossum *D. marsupialis* may also play a role in AVL transmission (Corredor et al., 1989a; Travi et al ., 1994).

### 1.2.2 Visceral Leishmaniasis

In Colombia, the occurrence of AVL fits well in type 2 and 3 patterns described in section 1.1.3.3, both with a high proportion of mild or subclinical infections. Although it is difficult to give precise figures on the annual incidence of AVL in the absence of accurate data, roughly 107 scattered cases have been diagnosed annually between 1944 and 1980. The majority of them came from the so-called upper Magdalena Valley (environ type 2; shaded area Figure 1.2). From this area was collected the earliest evidence of the presence of AVL in Colombia (Gast-Galvis 1944); a patient from San Vicente del Chucurí, in the north-eastern of the country. Several years then lapsed before studies on AVL were renewed in the area (Gómez-Vargas, 1965; Pérez-Norsagaray et al., 1970; García-Cuestas et al., 1970; Cantillo et al., 1970; Arciniegas & Duarte, 1976; Camacho et al., 1977). These studies were based mainly on reports of human cases, thus very little was gained on the vectors or the reservoirs of AVL. Although Gast & Renjifo (1944) enumerated the possible vectors in the San Vicente area, only *Lu. evansi* was specifically identified. In 1972, Osorno-Mesa et al., recorded *Lu. longipalpis* for the first time at three AVL foci in Melgar, Tocaima and La Peña. Some time later Marinkelle (cited by Camacho-Sánchez, 1978) collected this sandfly in Honda, close to

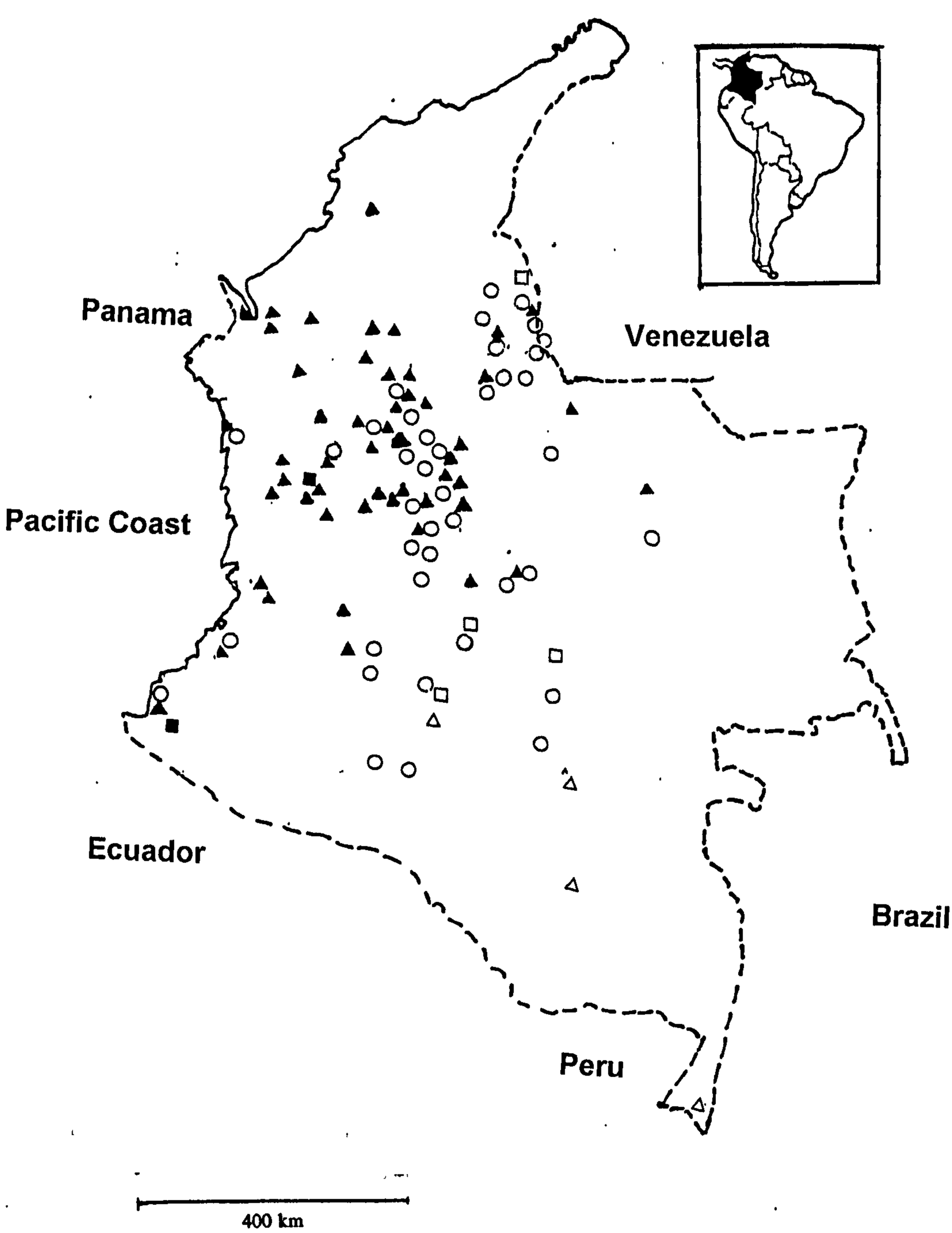


**Figure 1.1** Geographical distribution of *Leishmania* strains in Colombia. The most widely distributed are *L. braziliensis* (circles) and *L. panamensis* (black triangles). Other species have more restricted distributions: *L. guyanensis* (white triangles); *L. mexicana* and *L. amazonensis* (black and white triangles, respectively).

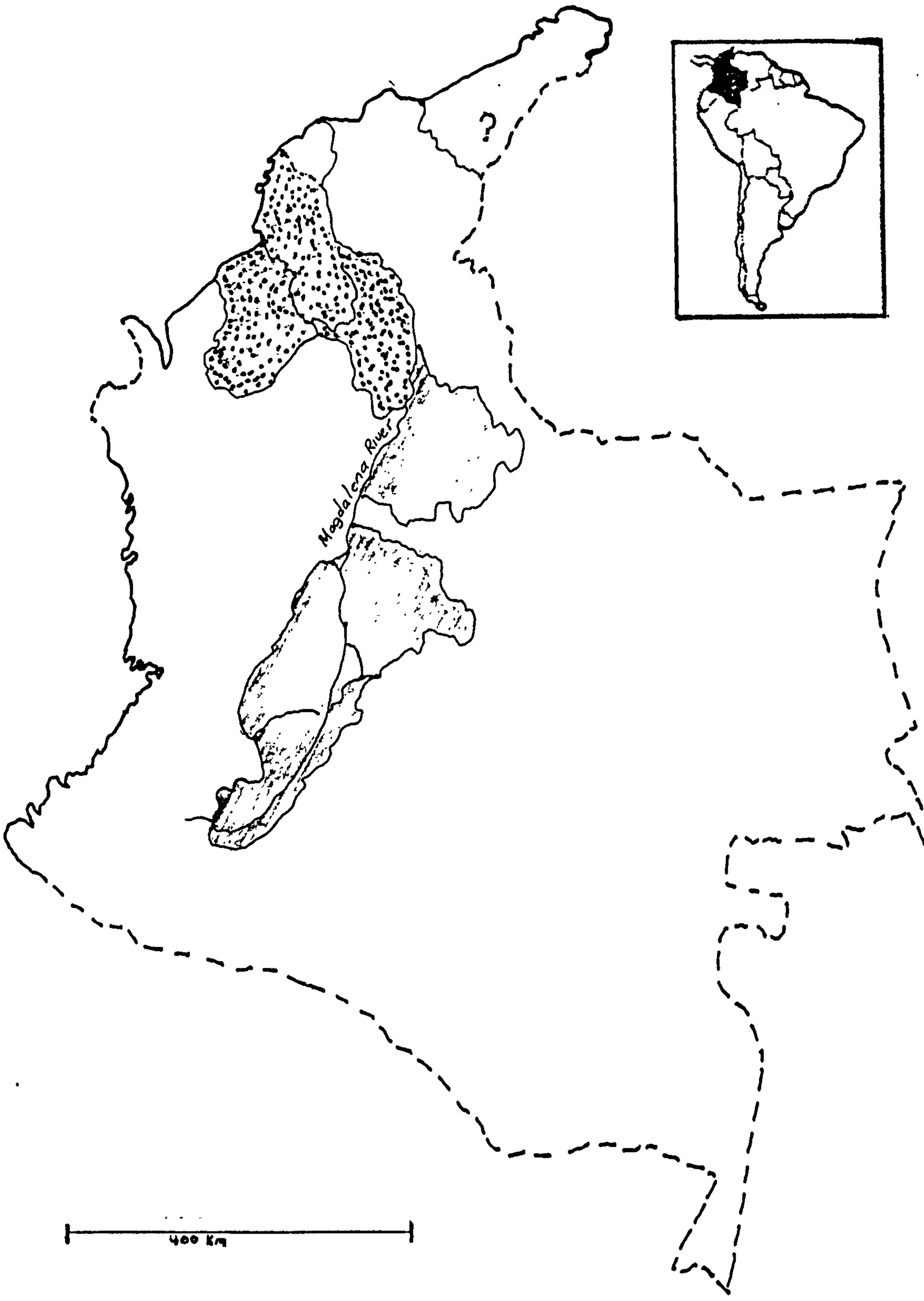


**Figure 1.2** Geographical distribution of *Leishmania chagasi* in Colombia. There are two defined foci: one along the Magdalena river (shaded area) and the second in the Caribbean coast (dotted area). **?** indicates an AVL focus where *Lu. evansi* and *Lu. longipalpis* are sympatric.

**Table 1.1:** Sandfly species incriminated or suspected of transmitting leishmaniae in Colombia.

| Sandfly | Parasite | Area/Ref. |
|---|---|---|
| Subgenus *Lutzomyia* | | |
| *Lu. (L.) longipalpis* | *L. chagasi* | Magdalena Valley[1] |
| *Lu. (L.) gomezi* (?) | *L. panamensis* | Antioquia[2] |
| Subgenus *Nyssomyia* | | |
| *Lu. (N.) trapidoi* | *L. panamensis* | Tolima[3], Pacific Coast[4,5] |
| *Lu. (N.) umbratilis* | *L. guyànensis* | Amazon Region[6] |
| *Lu. (N.) flaviscutellata* | *L. amazonensis* | Meta & Norte Santander[7] |
| Group: *Verrucarum* | | |
| *Lu. evansi* | *L. chagasi* | Caribbean Coast[8] |
| *Lu. spinicrassa* | *L. braziliensis* | Northeast Region[6] |
| *Lu. columbiana* (?) | *L. mexicana/L. panamensis* | Southwestern Region[9,10] |
| | *L. braziliensis* | |
| *Lu. youngi* (?) | *L. panamensis/ braziliensis* | Southwestern Region[11,12] |

[1] Corredor et al. (1989a); [2] Velez ID (Com. pers ); [3] Morales et al. (1981); [4] Travi et al. (1988); [5] Loyola et al. (1988b); [6] Young et al. (1987); [7] Grimaldi et al. (1989); [8] Travi et al. (1990); [9] Montoya et al. (in prep.); [10] Warburg et al. (1991); [11] Jaramillo et al. (1994); [12] Alexander et al. (1995).

Melgar village . However, was not until 1989 that a complete epidemiological evaluation was made of the El Callejón focus, a small but representative AVL rural community of the Magdalena Valley (Corredor et al., 1989a). These authors found a high natural infection rate (0.9%) of *Lu. longipalpis* with *L. chagasi* and isolated this parasite from patients, dogs and opossums. All of these results together with those of Morrison (1994), clearly show the specific association of *Lu. longipalpis* with AVL transmission in the Magdalena valley..

AVL environ type 3 (ie. areas where AVL exists without *Lu. longipalpis*) was detected for the first time in 1990 in an active focus of the drier Atlantic Coast centred on San Andrés de Sotavento. The sandfly *Lu. evansi* was found naturally infected with *L. chagasi* promastigotes (Travi et al., 1990; Travi et al., in press) in the absence of *Lu. longipalpis.* As yet there is no clear delimitation of the focus. However, subsequent reports of new cases of AVL from different neighbouring municipalities (Dr. ID Vélez, pers. comm.; Blanco-Tuirán et al., 1993), sharing similar entomological characteristics with the San Andrés de Sotavento

focus, suggests that AVL occurs throughout the whole area.

Now that a reasonable body of information is becoming available on the two major AVL foci in Colombia a brief description of each is made, with special emphasis on the entomological findings and the present status of sandfly studies. Unless otherwise stated, details of the Magdalena Valley and the Caribbean will be based on the El Callejón focus and the San Andrés de Sotavento focus, respectively.

1.2.2.1 The Upper Magdalena Valley focus (shaded area, Figure 1.2): This general area of more than 1,000km², is periodically flooded by the Magdalena river. Almost all of the reported AVL cases are from the right flood-plain of this river (departments of Huila, Tolima, Cundinamarca and Santander del Sur) to which *L. chagasi* appears to be restricted.

The El Callejón focus is situated in a semi-arid valley, at 400m above sea level, with a range of temperature between 24-32 °C and an average relative humidity of 82%. Houses and farms are scattered along the valley floor and along the principal stream. Houses are built of mud bricks or cinder blocks with plastered walls and corrugated metal roofs. There are small family farms planted with corn, sorghum, cotton, peanuts, yucca and citrus fruit. Domestic animals include dogs, cats, chickens, pigs, cattle, horses and donkeys. Native wild mammals are scarce but opossums (mainly *Didelphis marsupialis*) are abundant.

This small but endemic focus has been evaluated by an epidemiological cross-sectional survey (Corredor et al., 1989a) and, sometime later, by a prospective long term study on the natural prevalence of *L. chagasi* in *Lu. longipalpis* (Morrison, 1994). In brief, the authors found that *Lu. longipalpis*: (a) is the most abundant sandfly (on average 90.3% of the total captured); (b) is peridomestic and is adapted to the indoor human environment and (c) harbours *Lu. chagasi* promastigotes in nature. Although both studies provided epidemiological evidence implicating *Lu. longipalpis* in AVL transmission, it is important to note the wide variation in natural infection rates. While Corredor et al. (1989a) recorded an infection rate of 0.9% (n= 681) a value three times lower (0.3% of 3,811) was found in the second study (Morrison 1994). Also, the recovery of *L. chagasi* from humans, dogs and *D. marsupialis*-the suggested peri/intradomiciliar AVL reservoirs- (Corredor et al., 1989a) contrasted significantly with the almost nil preference for humans, dogs or opossums displayed by *Lu. longipalpis* (Morrison et al., 1993b). However, the authors think that the relatively small number of intradomiciliar *Lu. longipalpis* may play an epidemiologically significant role

in AVL transmission. It has been suggested that, as in other countries (Shaw & Lainson, 1987), there are sylvatic and domestic cycles in this area. The first cycle involving sylvatic populations of *Lu. longipalpis* and wild animals such as opossums (Corredor et al., 1989a; 1989b) which come to feed around houses at night where they are bitten by "domestic" *Lu. longipalpis*. Some of these sandflies develop the infection and subsequently transmit the parasite to dogs or humans. This initiates the peridomestic cycle with dogs as reservoirs.

1.2.2.2 The Caribbean focus (dotted area Figure 1.2): The actual delimitation, extension and general characteristics of this focus have been not established yet. However, according to the distribution of human cases, it appears that the area encompasses almost all the María Hills to the coastal zone of San Onofre in the north; the Sinú river swamps ("cienagas") in the south; the open savannas in the east and the hilly region of San Antero in the west. In contrast to the Magdalena valley, this is a semidry zone with an annual precipitation of 1,300mm and an average of temperature 25.5°C. The relative humidity is variable and influenced by its closeness to the sea.

Both cutaneous (produced by *L. panamensis*) and visceral leishmaniasis (due to *L. chagasi*) are endemic in the area (Vélez et al., 1988). However, VL has shown an unexpected increase in the number of cases. Since 1982, after the report of the first human AVL case in San Andrés de Sotavento, new cases have been found annually in this focus and neighbouring municipalities of Ovejas (Sucre) and El Carmén (Bolívar) (Dr ID Vélez, pers. comm.; Blanco-Tuirán et al., 1993). According to Servicio Seccional de Salud (Regional Health Service) the number of VL cases in San Andrés de Sotavento ranges from 7 to 13 per year. However, the number of clinical cases is suspected to be greatly under-estimated because of the adverse attitude of the inhabitants to outsiders, especially to medical and research personnel.

The continuing report of AVL cases in San Andrés de Sotavento (SAS), prompted the development of a wide multidisciplinary project to study the epidemiology of the disease. SAS is an aboriginal reserve of approximately 15,000 inhabitants of the Xinú tribe some 110km from the city of Montería in northern Colombia. There are two rainy seasons: March-April and August-September. However, the timing and severity of these seasons can vary considerably. The vegetation is scarce and typical dry secondary forest. The population is of an extremely poor socio-economic level and exists on a "cottage industry" of palm crafts and subsistence agriculture. During the early evening, many settlers usually sit outdoors. In some houses there are basic amenities such as latrines and electricity.

In the area, *Lutzomyia evansi* was found as the main sandfly species. As stated previously, earliest reports based on circumstantial observations, envisaged *Lu. evansi* as a potential vector in the transmission of the American visceral leishmaniasis (AVL) in atypical foci of Venezuela (Pifano & Romero, 1964) and Costa Rica (Zeledón et al., 1984). This assumption was, recently, bolstered after finding a *Lu. evansi* female naturally harbouring flagellates forms of *Leishmania chagasi*, in the AVL focus of SAS, an area free of *Lu. longipalpis*(Travi et al., 1990).After the first isolation of *L. chagasi* from *Lu. evansi*, two hamlets of SAS (El Contento and Vidales) were chosen for entomological studies. These concentrated on the sandfly fauna and natural infection rates. In all sandfly surveys, *Lu. evansi* was the predominant species displaying "anthropophilic" behaviour. Although *Lu. panamensis* and *Lu gomezi* were also found feeding on man they represented a small proportion of the sandfly population. Additionally, appreciable numbers of the two zoophilic species *Lu. trinidadensis* and *Lu. cayemensis* were found together with *Lu. evansi* in El Contento. Additionally, nine other *Lu. evansi* were found naturally infected. Two isolates were successfully identified as *L. chagasi* (Travi et al., in press).

Based on work in progress, it is thought that a similar epidemiological situation is occurring in some neighbouring localities of the Caribbean Coast (ID Vélez, comm. pers.) and in an AVL focus in an Andean town of Venezuela (Moreno & Oviedo, 1995). Thus, all these preliminary bits of evidence, suggest the existence of an AVL corridor, between the Caribbean Coast of Colombia and Venezuela, where *Lu. evansi* is the most likely vector.

## 1.3 Study Rationale

All the results above reinforce the previous suspicion that in the Caribbean area of Colombia *Lu. evansi* acts as an AVL vector in the absence of *Lu. longipalpis*. However, the important biological, physiological and ecological aspects which determine the importance of a sandfly species as a vector are unknown. Some of the outstanding questions concern larval and adult longevity (do adult females regularly survive long enough to take a second and infective blood meal?); sandfly movement (how much movement of *L. evansi* is there between resting sites and homes?); sandfly abundance (can differences in sandfly abundance be explained by differences in vegetation or in host availability?). Is there intradomiciliary transmission of visceral leishmaniasis?; host feeding preferences (does the ratio between different domestic animals affect the transmission of disease in the area? What is the actual contribution of dogs and opossums to *L. chagasi* transmission?); parasite development (how close is the parasite-sandfly relationship?) and not least, the taxonomic characterization of this sandfly should be determined.

These questions need two rather basic ideas in vector biology to be addressed: peridomesticity and anthropophily. What exactly do these terms mean?. The terms need clarification, both in general terms and in relation to this problem. Any sort of satisfactory approach to the problems listed above will rest on a deeper understanding of the dispersal and host preference of sandflies requiring exhaustive and thorough sandfly sampling. At the same time, it is necessary to note they are some of the most difficult parameters to be measured accurately.

## 1.4 Study Objectives

### 1.4.1 Principal Objectives

The aims of this study were:

To establish the role of *Lutzomyia evansi* (Núñez-Tovar) as the vector of *Leishmania chagasi* in the American visceral leishmaniasis focus of San Andrés de Sotavento.

To investigate those aspects of *Lu. evansi* biology which impinge on transmission and control.

### 1.4.2 Specific Objectives

In order to achieve the broad aims outlined above, the objectives were broken down into the following series of questions.

Abundance of the vector

Are there any seasonal or spatial differences in abundance?. What factors determine these differences?

Host vector contact

What are the host preferences of *Lu. evansi*, particularly in relation to the known reservoirs (dogs and opossum) and humans. When and where do the females bite?

Movement of the vector

How peridomestic is *Lu. evansi*? Is there substantial movement between the forest and houses? How is any movement related to bloodfeeding and oviposition?

Vector-Parasite relationships

What is the infection rate in Nature, is there any seasonal change in rate?.

Is the parasite *Leishmania chagasi* as well adapted to *Lu. evansi* as it is to the dominant New World vector *Lu. longipalpis*?.

Systematics of *Lu. evansi*

Given the disjuct distribution of *Lu. evansi*, is there any evidence for *Lu. evansi* being composed of more than one species?

By setting a series of questions it allowed the design of specific experiments or sets of observations to address them.

# CHAPTER 2

# GENERAL METHODS

The present study included both field and laboratory observations. The first part of this chapter consists of a brief description of the study area, the selection of the sampling sites criteria and the timing of study. In the second part, the basic procedures common to several different aspects are given. The more specific techniques used are described in the relevant chapters.

## 2.1 Study area description

Between 1992 and 1993, sandflies were collected from the field, mainly in El Contento, an Indian reservation occupied by approximately 300 members of the Xinú tribe, situated within the boundaries of San Andrés de Sotavento, some 110 km from the provincial capital of Montería in Córdoba (9° 09' N; 75° 31'W), northern Colombia (Figure 2.1). Short visits were made also to neighbouring villages (Balsal, Vidales, Gardenias, Vidalito, etc.) (Figure 2.1). Flies were also taken from other local municipalities (Sincelejo, Coveñas and Isla Fuerte) and other countries (Costa Rica and Venezuela) for biosystematic analyses.

Since the major part of this study was undertaken in the village of El Contento, the study area, the following description refers to this locality.

### 2.1.1 Meteorological information

The study area lies at the top of an escarpment at an elevation of 100m (Figure 2.2). Annual average rainfall is 1,300mm in two short rainy seasons, the first in March and April and the second in August and September (Figure 2.3), although the timing and volume of precipitation can vary considerably. The mean yearly temperature is 28°C.

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**Figure 2.1** Study area. The main study places are indicated by stars while arrows show the places where eventually sandfly collections were done. C= El Contento; G= Gardenias; V= Vidales; Co= Coveñas; B= Balsal; S= Sincelejo; IF= Isla Fuerte.

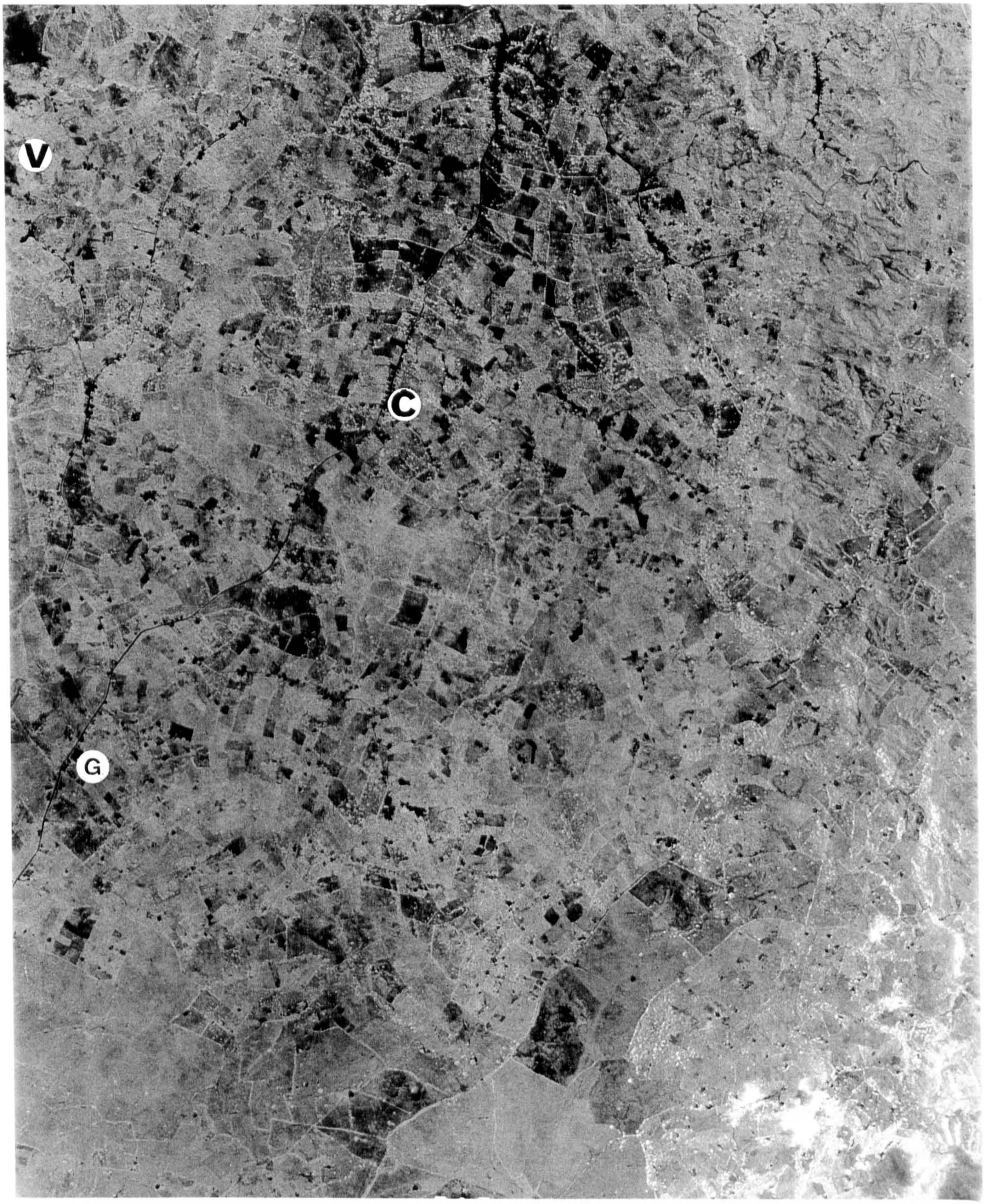


**Figure 2.2** Aerial photography showing part of San Andrés de Sotavento area. C= Contento; V=Vidales; G= Gardenias.

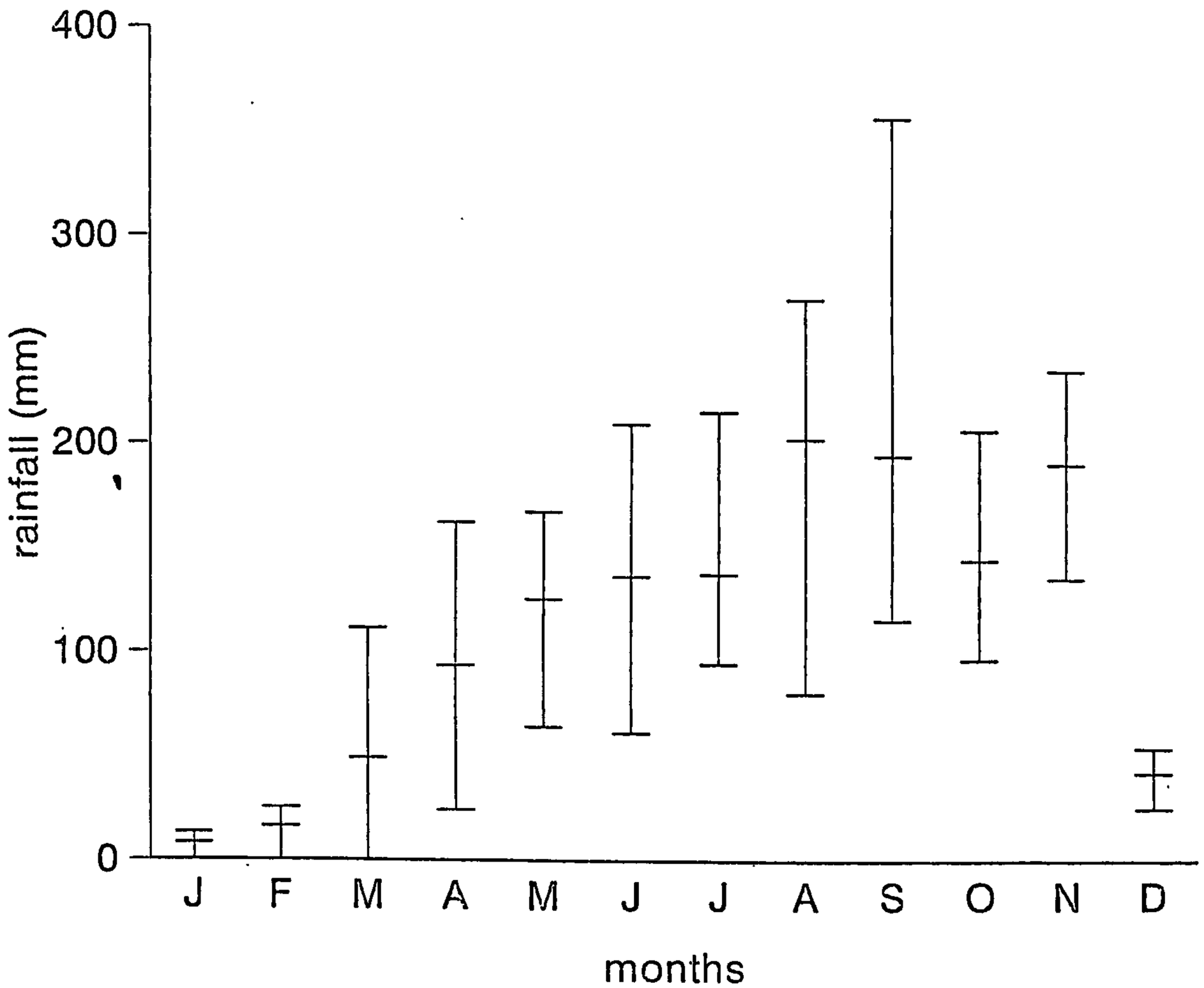


**Figure 2.3** Rainfall regime in the San Andrés de Sotavento area. Data (mean with maximum and minimum) plotted based on ten years compiled by the nearest meteorological station (Chinú Station).

2.1.2 Vegetation and animal composition

The typical vegetation consists of secondary dry-forest, considerably disturbed by human activity. There is no continuous forest canopy but scattered trees are present (Figure 2.4). An inventory of the main trees present in the area follows: oak (*Quercus* sp.); ceiba (*Ceiba pentandra*); "guasimo" (*Guamuza ulmifolia*); "totumo" (*Crescienta cujete*); "Caucho" (*Ficus* sp); "tamarindo" (*Tamaridus indicus*); *Melicocca bijuca*; "mataratón" (*Gliricidia sepium*) (see Figure 2.5).Deforestation of the natural forest has been driven by the demand for fuelwood and to provide plots for the production of cash crops (plantain, cassava and maize) and pasture lands (cattle, pigs and donkeys). The wild fauna is depauperate and native wild mammals (eg. foxes) are scarcely seen. Conversely, the highly adaptable opossum (*Didelphis marsupialis*) and the black rat (*Rattus ràttus*) are abundant around human dwellings. A two-week trapping survey during the study period, showed that the murid *Zygodontomys*

**Figure 2.4** Landscape of the San Andrés de Sotavento area.

*brevicauda* and *D. marsupialis* were the predominant sylvatic species, with the rodents *Proechymis canicollis*, *Heteromys anomalus* and *Oryzomys concolor* also present in the area. The red-headed lizard (*Anolis* sp.) is common both inside and outside houses. The most frequent domestic animals are dogs, fowl (chickens, turkeys), donkeys and pigs. Cows and lambs are also present but in fewer numbers.

### 2.1.3 Sociological information

Dwellings are primitive, wood-framed with mud-plastered walls and palm-thatched roofs. Most have porches in which the inhabitants sit at sunset and during the early evening (Figure 2.6). The housing density is variable, although a main nucleus of houses exists some are scattered and isolated up to 1km from their nearest neighbour. The population is of an extremely poor socio-economic level. Plantations are usually situated more than 200m from houses. Animal pens are not frequent and usually the animals are tethered and sleep around houses (Figure 2.7). A few houses have basic amenities such as latrines and electricity.

**Figure 2.5** Typical trees found around human settings in the SAS area.

**Figure 2.6** A typical Indian Xinú house. This type of housing does not prevent sandfly entrance: walls usually have interstitial gaps and the ceiling is not covered.

**Figure 2.7** Cows and pigs are not very abundant in the area. Animals wander during the day time but at evening they are tethered to trees where they spend the night.

2.1.4 Epidemiological information

Detailed information on the severity and importance of the disease is not available. Since data collected by the local Health Services is not considered reliable, it is difficult to establish the actual incidence and prevalence rates of the disease. However, it is known that the area is endemic for both visceral and cutaneous leishmaniases due to *L. chagasi* and *L. panamensis*, respectively (Travi et al., 1990). The most reliable information, based on the isolation and characterization of *Leishmania* parasites from patients and serology, has been collected during the past five years by a CIDEIM-Universidad de Antioquia-Servicio de Salud team, but this represents only 10% of the SAS population. These data have revealed that overall cases of VL are more abundant than was previously thought and almost all restricted to the under three-old-year group. In contrast, cutaneous cases are commonest in the over 14-year-old group. Annually, 12 new VL cases, with geographical histories indicating that they have acquired the disease at home, are recorded in the area. Mild or asymptomatic infections are known to occur in patients (Dr. G. Palma, pers. comm). Interpretation of the human and dog seroprevalence (IFAT) and Montenegro tests is difficult. The number of skin test positive patients increases directly with age, and no correlation between dog and human infections has been established. However, based on the analysis of data from these tests, Travi et al. (in press) considered two areas: one as "high" (Vidales) and other as "low" (El Contento) endemicity in the focus. El Contento although is regarded as a "low" endemic locality has "high" parasite seroprevalence in dogs. Interestingly, when the position of individual VL cases in SAS are mapped, it appears that the majority live in houses in the forest area ie. at the periphery of the village. However, no statistical significance has been found in this association.

## 2.2 Study structure

2.2.1 Definitions and assumptions:

For the purpose of the present study, the following definitions are used:

* "House": a building, which has at least 4 inhabitants, dog, adobe or wooden walls, thatched roofs, most lack electricity.

* "Few domestic animals": When the total number of domestic animals is eight or less individuals.

* "High domestic animals": When the total number of domestic animals is equal or more than

9 individuals.

* "Close to forest": a house which is less than 5 metres from the forest.

* "Far to forest": a house which is more than 5 metres from the forest.

* "Peridomiciliary capture": sandfly captures at least 5 metres but no more than 50 metres away from a house.

* "Intradomiciliary capture": sandfly captures inside rooms and in an area less or equal to 5 metres from a house.

Also, it was assumed that sandfly populations were not affected by any sampling procedure applied in the area, ie. "trapping-out" was not a significant effect.

2.2.2 Timing of study:

Based on seasonal and entomological data previously recorded (Travi et al., in press and the author, unpublished pilot study), six bimonthly periods for sampling were chosen. Thus, during the field work at least one period at the beginning of the season with predominantly young sandflies (with reasonable expectancy of longevity), and another at the end with mainly old females (with high potential of transmission) were covered. Therefore, dry and rainy periods were also covered during the study.

## 2.3 Entomological procedures

2.3.1 Sandfly composition and abundance studies:

As a first approximation to study the sandfly populations in San Andrés de Sotavento, time spaced collecting procedures were used to determine species composition and with some appropriate refinements, to estimate their abundance (Morris, 1960).

2.3.1.1 Sticky trap captures: Non-attractant sticky traps have been used most often in the Old World (eg. Dergacheva et al., 1979; Quate, 1964; Rioux et al., 1982; Yuval, 1991; Asimeng 1991; Basimike et al., 1991) and to a lesser extent in some dry areas in the Americas (Cameron et al., 1994; 1995; Ferro et al., 1995) to estimate sandfly composition and densities. According to Quate (1964) and Dergacheva et al. (1979) it is the best method available for making random samples of sandflies in wild and in human settlements. During the present study, sticky traps were made by immersing numbered sheets of bond paper (20cm x 30cm) in castor oil, at least seven days before use.

For intradomiciliary sticky trap collections, traps were hung using strings and plastic

clips inside houses, ensuring exposure of both sides. Inspection and replacement of the traps was done on a daily basis. Caught flies were removed using a needle and rinsed in weak shampoo solution before clearing. Thus, unfed females and male sandflies were cleared in 1:1 acid lactic: phenol solution. Identification was done directly in the clearing solution, following Young's key (1979). Specimens which could not be identified reliably by this method were slide mounted in Berlese medium (Lewis, 1974). Fed flies were preserved for blood meal analysis (see Chapter 4). When required, females were physiologically age graded following the criteria of Ready et al. (1984).

2.3.1.2 Searching in resting places: This represents another reliable method of estimating insect composition and abundance. According to Service (1993), it usually provides a more representative sample of the population as a whole than most other methods. Direct search and collection from resting places, has been useful in the study of *Phlebotomus* (Hati et al., 1987; Foster, 1972) and *Lutzomyia* (Alexander, 1987; Memmott, 1992; Ferro et al., 1995) populations. In the present study, resting sandflies (including freshly engorged) were actively searched for both indoors (walls and crevices) and outdoors (tree-holes, buttresses, leaves) and other sites suspected to be sandfly resting places. Direct searches were performed routinely between 06:00-07:00h for a fixed period of time (10min/place); this provided a standard effort, allowing relative estimates of sandfly abundance.

2.3.1.3 Light-baited traps: A modification of the original Shannon trap (Shannon, 1939) was used to catch sandflies for a variety of purposes. The actual trap was made of cotton sheeting and consisted of a central cubicle (1.5m high x 1.2m wide and 1.5m long) with shorter side cuboid compartments (0.5m high x 1.2m wide x 0.5m long) attached at the top of the trap. The trap was always erected in forest settings, baited with two battery powered fluorescent tubes placed in the middle of the central compartment (Figure 2.8). Sandflies were collected either on the outside or inside compartments, usually between 19:00 and 22:00h. Captures by this means, were made in El Contento; Vidales; Balsal and Gardenias (Figure 2.1) to determine sandfly composition. Also additional Shannon trap collections were made for isozyme studies and sandfly colonization.

At the beginning of the study (November 1992- January 1993), CDC battery-operated traps were used to compare the intra and peridomiciliar sandfly composition. However, their use was discontinued due to low numbers of flies collected and consistent thieving.

**Figure 2.8** A human baited Shannon trap in operation

# CHAPTER 3

# SANDFLY COMPOSITION AND ABUNDANCE PATTERNS

## 3.1 Background

Since epidemiologically *Lu. longipalpis* is recognized as the main vector of *L. chagasi*, its bionomics has been extensively observed and studied from the 1930's (Chagas et al., 1937, 1938) until recently (Morrison, 1994). Conversely, given the freshly suspected involvement of *Lu. evansi* in the transmission of AVL, the available body of information about the bionomics of this sandfly is fragmentary (cf. Pifano & Romero 1964; Young 1979; Zeledón et al., 1984; Zeledón et al., 1989; Feliciangeli et al., 1992; Travi et al., in press).

This chapter reports and discusses the results of basic entomological studies on hematophagous species composition of the SAS focus, sandfly seasonal variation, especially of *Lu. evansi* and spatial distribution. All this information was directed to delimit the ecological boundaries of the disease in AVL foci where *Lu. evansi* is the suspected vector.

## 3.2 Methodology

### 3.2.1 Species composition and relative abundance

3.2.1.1 General survey of SAS and surrounding areas: To determine the species present in the neighbouring areas (to put the SAS sampling in context) ad hoc sandfly collecting was carried out by a variety of methods including searching resting places, sticky traps and Shannon traps (see Chapter 2) in three other municipalities: Sincelejo and Coveñas, some 40 and 60km away from SAS to the north-west and on Isla Fuerte, an island off the coast (see Figure 2.1).

3.2.1.2 Species composition and relative abundance in SAS: The species composition in the main focus was determined by pooling the results from all the main sampling methods ie. sticky traps, human bait, direct search in resting places and cone traps throughout the year.

3.2.1.3 Between village variation: These two basic parameters were determined by time spaced sampling collections using sticky traps in the villages of Gardenias, El Contento, Vidales and El Balsal (Figure. 2.1). These localities were selected on the basis of accessibility and history of human or dog VL cases and because the majority of their houses follow the general housing pattern described in Chapter 2 (section 2.1.3), ie. small unit with mud plastered walls and thatched roofs. The differences in sandfly composition between these villages were established by simultaneous sticky trap collections, covering 12 days of both the dry and wet seasons. Due to logistical problems complete data were not obtained from Balsal, hence the results are based on the total of catches of the remaining three hamlets. In each village, a total of 40 sticky traps were randomly located in the extra domiciliary areas around two houses. These collections were done for alternate 4-day cycles, traps being inspected and changed every time.

3.2.2 Seasonal distribution

In an attempt to represent all dwellings of the El Contento village eight sandfly collecting places (ie. houses) were selected. Houses were matched on their relative closeness to the forest and the number of domestic animals, forming a total of four environs. Thus, houses 1 and 2 were away from forest but had a high number of domestic animals. The reverse situation was exhibited by houses 4 and 8 (ie. they were close to the forest and had a lot of domestic animals). Finally, houses 3 and 5 were farther from the forest and had fewer animals than houses 6 and 7. Figure 3.1 shows a schematic representation of the four studied environs.

Temporal variations in sandfly densities were determined by longitudinal sticky trap sampling over a year. Initially, sticky trap catches were attempted in both intra and extra domiciliary settings in the eight selected houses in El Contento area. However, the adverse reaction of the local people prompted the withdrawal of this technique from the extra domiciliary settings. Instead, a day-light search for flies in the vegetation around houses was used as an alternative sampling method. This was carried out in the morning (06:00h) by a

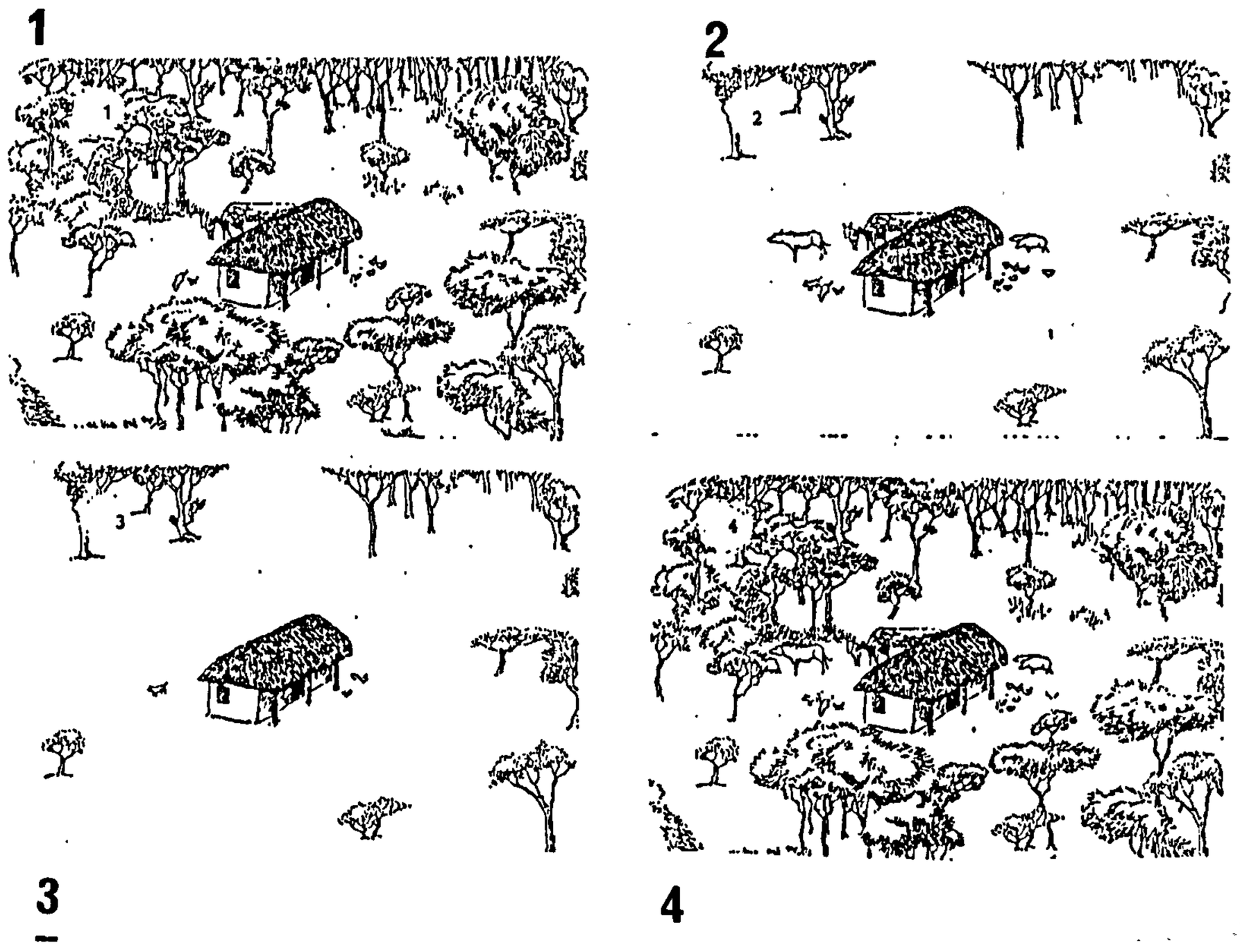


**Figure 3.1** The four type of environments present in the SAS area (details in the text)

standard period of 40min/house of direct collection of sandflies in all conceivable resting places around the house, especially big trees and shaded places. In order to reduce bias associated with sample size and location, and to guarantee reliable comparisons, for each house the surrounding area of a 15m radius was divided into four quadrants. A total of four houses (1,2, 4 and 5) were selected for this study. In each house, the vegetation of each quadrant was inspected by a standard 10-minute direct-collection on a daily basis for five consecutive days. The procedure was repeated in April, June, July August and November 1993. Tree species were identified and their relative sandfly abundance compared.

### 3.2.3 Microhabitat

3.2.3.1 Catches in forest and non-forest areas: To determine whether or not sandfly composition and abundance varied according to location of a house relative to the forest, sets of 20 sticky traps/day/house were evenly distributed inside eight houses, four in the forest and

four in the non-forest area. Thus, a total of 1,280 traps were run over eight consecutive days each month between January and November 1993. A house which is less than 5 metres from forest is considered "close to forest" while a house more than 5 metres from forest is regarded as "far to forest".

3.2.3.2 Vertical distribution: To establish if there were differences in the height that sandflies moved or rested in the intra domiciliary setting, a set of 10 sticky traps was set 0.1- 0.15m above the ground and a further set of 10 traps approx. 1.8 - 2.0m above ground, both inside and outside bedrooms of each house. Thus, a total of 640 traps at each height were run per month, between January and November 1993.

3.2.4 Daily activity pattern

The daily sandfly pattern was determined by indoor collection of flies landing on humans and by extra domiciliary catches with Shannon traps in the forest (evening activity only). Human bait catches for 50 min/hour were made for eight nights as explained in Chapter 4 (section 4.2.4), while Shannon trap catches were carried out at the edge of the forest (ie. sampling forest and non-forest areas) between 19:00 and 21:00 hours. Thus, during this two-hour period, three people made sandfly catches at five minutes intervals, separating catches in labelled vials. Data on temperature and relative humidity were recorded parallel to the sampling. The whole procedure was repeated for four consecutive nights, one carried out in March-April 1994 (dry season) and the other in August-September 1994 (rainy season) producing independent replicates per season.

3.2.5 Analysis of data

The diversity of the catches in each area was calculated using the Shannon-Weaver Index (Magurran, 1988; p. 34). This is defined as:

$$H = - \Sigma\ p_i \ln p_i$$

where $p_i$ is the proportion of a particular species in a sample which is multiplied by the natural logarithm of itself. H is derived by summing the product for all species in the sample. Sandfly diversity indexes were compared between localities and by season using t-tests, which requires calculation of the variance for the H values according to Magurran (1988; p. 37).

Questionnaires previously prepared in DBASE III (Ashton Tate, Torrance, CA) were used to record data obtained by sticky trap and human bait catches. In all cases, data were double checked, then examined for normality and, when necessary, were normalized by log transformation. Sticky trap and human bait catches were converted to sandfly rates (geometric means), ie. number of sandflies collected per trap/month and per person/hour, respectively. Analysis of variance using a Statistical Analysis for Social Sciences (SAS, 1982) general linear models procedure was employed, testing the hypotheses that the mean numbers of flies obtained from each area, stratum or position were equally distributed. Categories were included in the model when the calculated F value was less than the tabular F value at $p= 0.01$. When non-parametric analysis was used, the Kruskal Wallis $\chi^2$ test was selected for comparisons using the Epi-Info 5.0 statistical package.

## 3.3 Results

### 3.3.1 Species composition and relative abundance

3.3.1.1 General survey: The dates, method of capture, numbers and sandfly composition of

**Table 3.1** Sandfly composition and total numbers of flies caught in ad hoc sampling procedures in neighbouring localities of San Andrés de Sotavento in 1992-93. (hb=human bait; rp= resting places; sh=shannon trap; T=total).

| | Sincelejo[1] | | | | Coveñas[2] | | | | Isla Fuerte[3] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | hb | rp | sh | T | hb | rp | sh | T | hb | rp | sh | T |
| *Lu. evansi* | -- | -- | -- | -- | 1 | 2 | -- | 3 | 10 | 22 | -- | 32 |
| *Lu. cayennensis* | -- | 6 | -- | 6 | 4 | 28 | -- | 32 | 3 | 14 | -- | 17 |
| *Lu. trinidadensis* | -- | 14 | -- | 14 | 0 | 5 | -- | 5 | 5 | 70 | -- | 75 |
| *Lu. dubitans* | -- | 0 | -- | 0 | 0 | 0 | -- | 0 | 0 | 0 | -- | 0 |
| *Lu. atroclavata* | -- | 4 | -- | 4 | 0 | 3 | -- | 3 | 0 | 7 | -- | 7 |
| *Lu. rangeliana* | -- | 0 | -- | 0 | 0 | 1 | -- | 1 | 0 | 0 | -- | 0 |
| *Lu. panamensis* | -- | 0 | -- | 0 | 0 | 0 | -- | 0 | 0 | 0 | -- | 0 |
| *Lu. gomezi* | -- | 0 | -- | 0 | 0 | 0 | -- | 0 | 1 | 0 | -- | 0 |
| **TOTALS** | -- | 24 | -- | 24 | 5 | 39 | -- | 44 | 19 | 113 | - | 129 |

[1] September 1992; [2] January 1993; [3] January 1994.

each locality sampled are shown in Table 3.1. Although *Lu. evansi* was present in two of the localities, it was much less abundant than *Lu. cayennensis* and *Lu. trinidadensis* in Coveñas and Isla Fuerte, respectively.

3.3.1.2 Species composition and relative abundance in SAS: Ten species were found in the area. The numbers and percentages of sandfly species caught by four methods in the villages of San Andrés de Sotavento, between January and December 1993 are given in Table 3.2. From a total of 4,760 female and 5,237 male *Lutzomyia* collected, *Lu. evansi* was always the most abundant species, representing 86.04% of the overall catch (Table 3.2). The abundance of the other species varied with place, method and time of collection, with either *Lu cayennensis* or *Lu. trinidadensis as* the second most abundant species in the collections. The combined catches of these two species represented roughly 10.0% of the captures. *Lu. rangeliana, Lu. dubitans, Lu. gomezi, Lu. panamensis, Lu. shannoni, Lu micropyga* and *Brumptomyia beaurperthuyi* constituted the remaining small fraction of the total sandfly population. Interestingly, *Lu. longipalpis* was not found by any means in the sampled villages. Sandfly collections with sticky traps were characterized by an almost equal sex ratio in all environments, collections using either human or animal baits showed predominance of female flies while those done by direct search were male biased (Table 3.2). Unrecorded but variable number of other hematophagous arthropods included species of *Anopheles* prob. *punctimacula, Aedes* spp., *Culex* spp., *Psorophora ferox, Ctenocephalides canis* and *Amblyomma* sp.

3.3.1.2 Between village variation: The results of the two 12-day sampling periods done simultaneously in Gardenias, El Contento and Vidales to measure differences in sandfly abundance during dry and rainy seasons are given in Table 3.3. A total of 2,800 sticky traps were used. Comparison of diversity values (H) between localities indicates a clear trend, these values were always significantly higher in El Contento whatever the season (Table 3.4). In Vidales, a dramatic but surprising reduction in the H values was noted between dry and wet season period (Table 3.3), which were statistically different at $p < 0.001$ ($t = 5.70$; $df = 135$). Conversely, no differences between seasons were found in El Contento or Gardenias (Table 3.4).

In El Contento, *Lu. micropyga* and *B. beauperthuyi* were the only species absent during the dry season. Species such as *Lu. micropyga, Lu. shannoni* and *B. beauperthuyi*

**Table 3.2** Number, percentages (%) and sex ratios (female = F and male M) of *Lutzomyia evansi* and other species of *Lutzomyia* caught by diverse methodologies in the San Andrés de Sotavento AVL focus-Córdoba: Colombia , between January and December 1993.

| | *Lutzomyia evansi* | | | Other *Lutzomyia* spp. | | | GRAND TOTAL | | |
|---|---|---|---|---|---|---|---|---|---|
| Capture Method | F | M | Total | F | M | T | F | M | T |
| Sticky traps | 738 | 815 | 1553 | 31 | 67 | 98 | 769 | 882 | 1651 |
| | (44.7) | (49.4) | (94.0) | (1.9) | (4.0) | (5.9) | (46.6) | (53.4) | (100) |
| sex ratio | | | 0.90 | | | 0.46 | | | 0.87 |
| Human bait | 993 | 131 | 1124 | 82 | 54 | 136 (10.8) | 1075 | 185 | 1260 |
| | (78.8) | (10.4) | (89.2) | (6.5) | (4.3) | | (85.3) | (14.7) | (100) |
| sex ratio | | | 7.6 | | | 1.52 | | | 5.8 |
| Cone trap | 335 | 166 | 501 | 22 | 15 | 37 | 357 | 181 | 538 (100) |
| | (62.3) | (30.9) | (93.1) | (4.0) | (2.8) | (6.9) | (66.4) | (33.6) | |
| sex ratio | | | 2.0 | | | 1.46 | | | 1.97 |
| Direct search | 2157 | 3267 | 5424 | 402 | 722 | 1124 | 2559 | 3989 | 6548 |
| | (32.9) | (49.9) | (82.8) | (6.0) | (11.0) | (17.2) | (39.0) | (61.0) | (100) |
| sex ratio | | | 0.66 | | | 0.56 | | | 0.64 |
| TOTAL | 4223 | 4379 | 8602 | 537 | 858 | 1395 | 4760 | 5237 | 9997 |
| | (42.2) | (43.8) | (86.0) | (5.4) | (8.6) | (14.0) | (47.6) | (52..4) | (100) |
| | | | 0.96 | | | 0.62 | | | 0.90 |

**Table 3.3** Sandfly composition, relative abundance (number of flies/m2 sticky paper) and Shannon-Weaver index for Gardenias (G), El Contento (C) and Vidales (V) villages of San Andrés de Sotavento-Córdoba: Colombia) .

| | RAINY SEASON | | | DRY SEASON | | | TOTAL | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **G** | **C** | **V** | **G** | **C** | **V** | **G** | **C** | **V** |
| *Lu. evansi* | 89 | 185 | 213 | 31 | 67 | 65 | 120 | 252 | 278 |
| | 0.190 | 0.396 | 0.456 | 0.066 | 0.144 | 0.139 | 0.257 | 0.540 | 0.596 |
| *Lu. trinidadensis* | 18 | 40 | 16 | 6 | 6 | 2 | 24 | 46 | 18 |
| | 0.039 | 0.086 | 0.034 | 0.013 | 0.013 | 0.004 | 0.051 | 0.099 | 0.039 |
| *Lu. cayennensis* | 13 | 24 | 35 | 12 | 15 | 0 | 25 | 39 | 35 |
| | 0.028 | 0.051 | 0.075 | 0.026 | 0.032 | — | 0.054 | 0.084 | 0.075 |
| *Lu. gomezi* | 4 | 9 | 33 | 0 | 3 | 11 | 4 | 12 | 44 |
| | 0.008 | 0.019 | 0.070 | — | 0.006 | 0.024 | 0.008 | 0.026 | 0.094 |
| *Lu. panamensis* | 3 | 18 | 34 | 0 | 5 | 3 | 3 | 23 | 37 |
| | 0.006 | 0.039 | 0.073 | — | 0.011 | 0.006 | 0.006 | 0.049 | 0.079 |
| *Lu. rangeliana* | 2 | 9 | 5 | 0 | 5 | 0 | 2 | 14 | 5 |
| | 0.004 | 0.019 | 0.011 | | 0.011 | — | 0.004 | 0.030 | 0.011 |
| *Lu. micropyga* | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |
| | — | 0.015 | — | — | — | — | — | 0.015 | — |
| *Lu. dubitans* | 0 | 3 | 5 | 0 | 1 | 0 | 0 | 4 | 5 |
| | — | 0.006 | 0.011 | — | 0.002 | — | — | 0.008 | 0.011 |
| *Lu. shannoni* | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| | — | 0.006 | — | — | — | — | — | 0.006 | — |
| *B. beauperthuyi* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| | — | 0.004 | — | — | — | — | — | 0.004 | — |
| **Shannon-Weaver** | **1.02** | **1.36** | **1.25** | **0.89** | **1.17** | **0.66** | **1.01** | **1.33** | **1.14** |

were never collected in Gardenias nor in Vidales. *Lu. dubitans,* although present in Vidales, was not recorded in Gardenias and *Lu. rangeliana* though present during the wet season was totally absent in both localities during the dry season. The anthropophilic *Lu. gomezi* and *Lu. panamensis* were not detected in Gardenias in the dry season though they were recorded in Vidales at the same period.

**Table 3.4** t test comparisons of the Shannon-Weaver (H ) values for three villages in the San Andrés de Sotavento area.

| **Season: Village Comparison** | **t** | **df** | **p** |
|---|---|---|---|
| Rainy | | | |
| Gardenias: El Contento | 3.71 | 282 | 0.001 |
| Gardenias: Vidales | 2.50 | 236 | 0.02 |
| El Contento: Vidales | 1.92 | 600 | NS |
| | | | |
| Dry | | | |
| Gardenias: El Contento | 3.32 | 149 | 0.002 |
| Gardenias: Vidales | 2.22 | 129 | 0.05 |
| El Contento: Vidales | 4.91 | 182 | 0.001 |
| | | | |
| Rainy: Dry | | | |
| Gardenias: Gardenias | 1.56 | 152 | NS |
| El Contento: EL Contento | 1.76 | 192 | NS |
| Vidales: Vidales | 5.70 | 135 | 0.001 |

3.3.2 Seasonal abundance pattern

From 10,800 traps run during 80 simultaneous sticky trap sessions, a total of 1,877 sandflies was caught. Since *Lu. evansi* represented 82.3% of the total captures it was the only species from which a clear seasonal pattern could be detected; a positive association was found with rainfall (Figure 3.2). Although intra domiciliary sampling began in January 1993, it was not until the beginning of the rains in April that sandfly presence indoors was detected. Thereafter, the largest mean value of flies was reached (0.45 flies/trap). Through the following

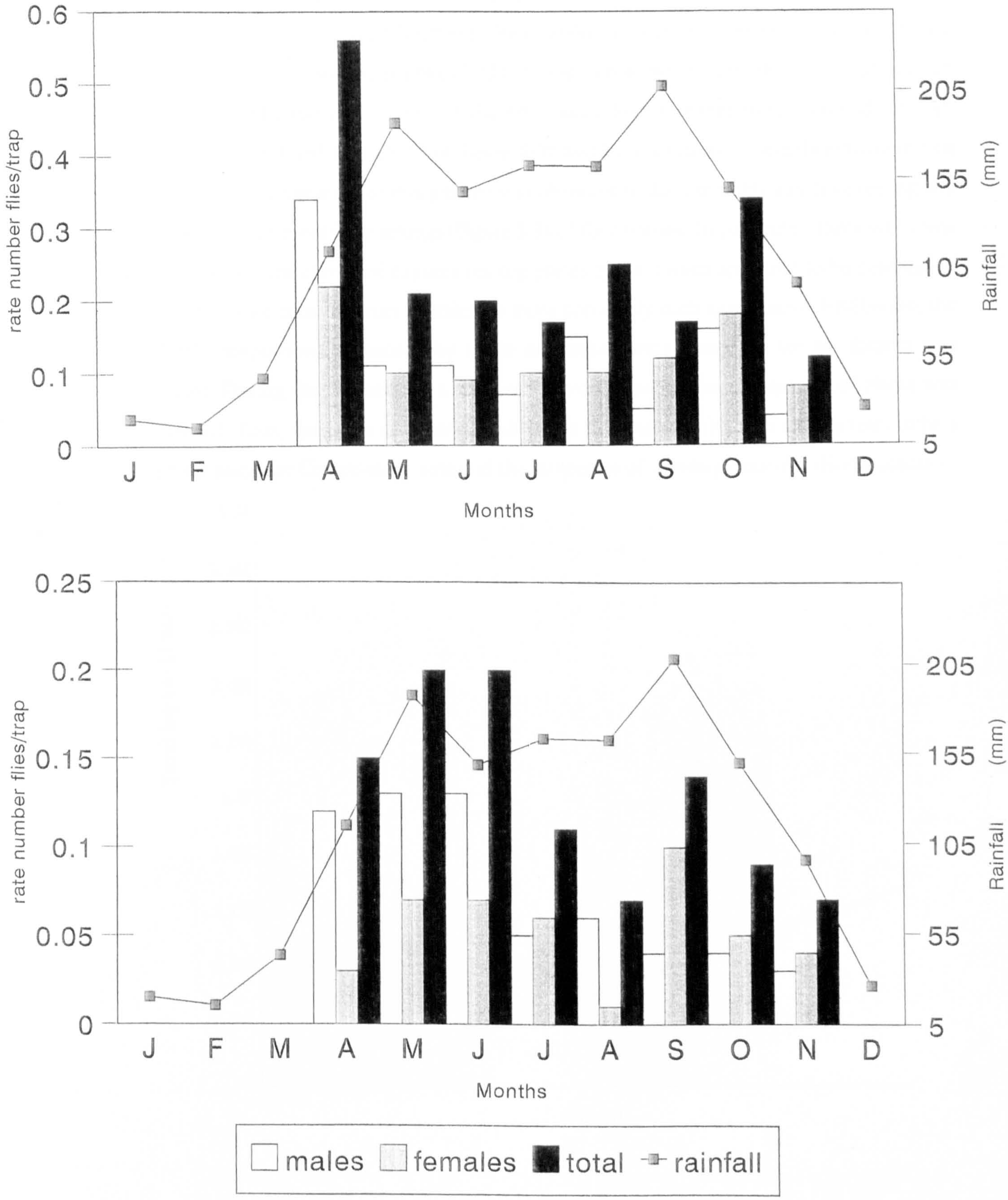


**Figure 3.2** Seasonal abundance of *Lu. evansi* measured by monthly sticky trap captures. Top: forest area; bottom: open area.

months, the sandfly population decreased, apparently being affected by a short but very windy and rainy period in July (0.14 flies/trap). Then, sandfly activity experienced a gradual increase till October, when a secondary peak (0.22 flies/trap) is reached. Finally, the sandfly population declined rapidly towards the end of the dry season. Few sandflies were recorded between November 1993 and January 1994, being followed by a cessation of sandfly activity during February. Slight variation to this pattern was observed in the bimonthly day-time resting site catches in extra domiciliary settings (Figure 3.3) of four houses. In particular, there was some indication that the choice of daytime resting places by *Lu. evansi* appeared to be determined by some biotic cues. In order to examine more accurately such aggregative distribution, the sandfly proportions, measured by direct aspiration, were compared for six distinct tree species. During the dry season, a rank in the proportion of flies per species of plants was observed. Thus, treeholes of *Guamuza ulmifolia* (Sterculiaceae) which also appears to be a nesting place for *Camponotus* ants and the buttresses of *Ceiba pentandra* (Bombacacae)

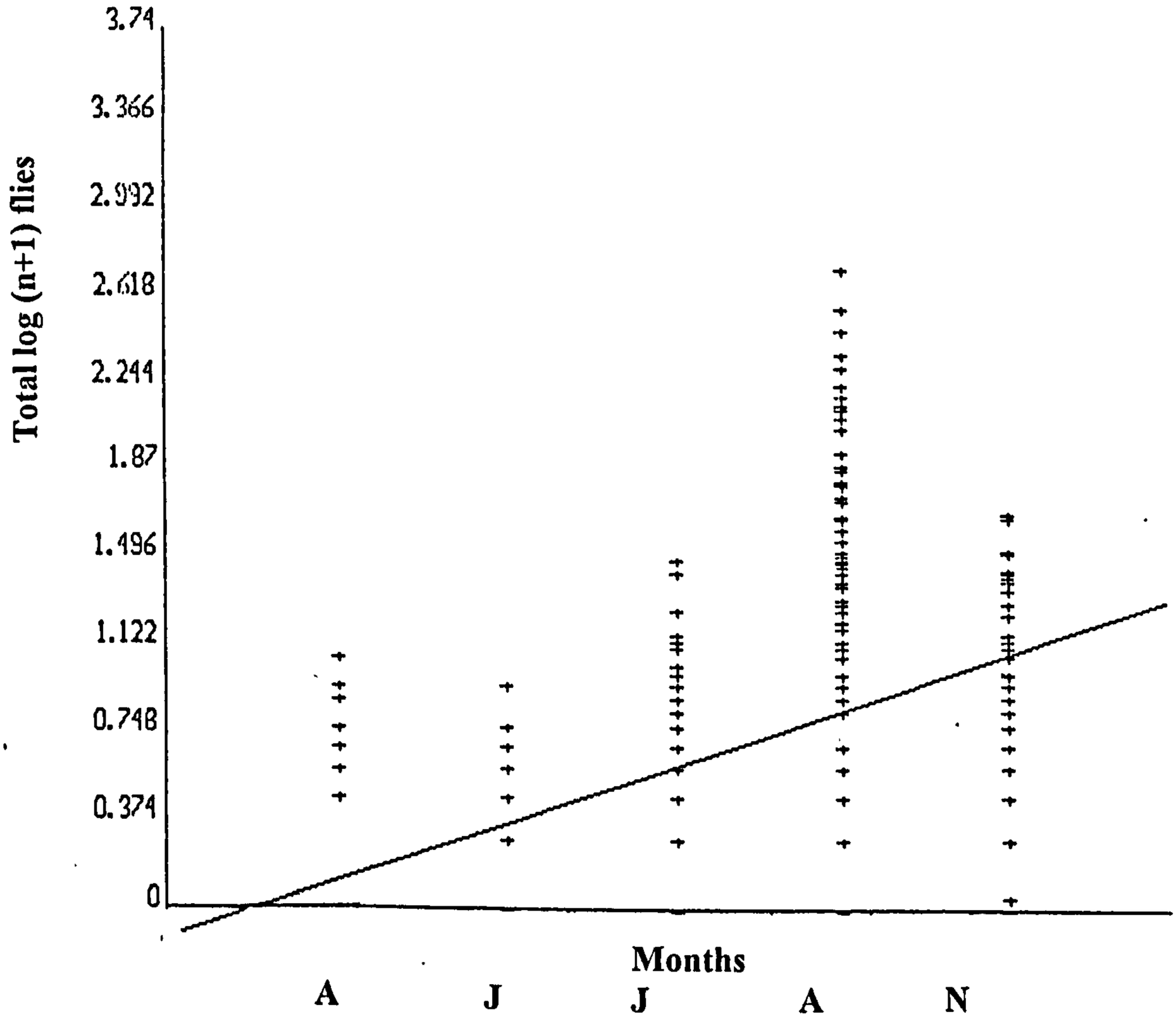


**Figure 3.3** Seasonal pattern exhibited by resting *Lu. evansi* in El Contento village.

provided the most attractive resting site for sandflies, mainly *Lu. evansi*. Sandflies were also seen, though to a lesser extent, resting on *Ficus* sp. and barks of *Gliricidia sepium* and the oak *Querqus* spp. This pattern, although consistent throughout the study, varied with the seasonal sandfly peaks (eg. June and August). At these periods, after some heavy rains no defined preferences were detectable. Flies were found resting on the barks of almost any tree, even in those species previously reported as 'unfavourable' (eg. leaf-axils of some palms trees). It was noted, but not quantified, that in the morning sandflies moved up the trunks, apparently avoiding the excess water at the bottom of the trees. An event which drew our attention was that despite nocturnal activity of both female and male *Lu. evansi* in fruits, branches and leaves of *Crescienta cujete* (Figure 3.4), very few resting flies were seen in this tree species.

**Figure 3.4** Detail of the fruit and leaves of *C. cujete*. At night *Lu. evansi* individuals were seen moving around the fruit, branches and leaves of the tree, however, few remain resting during day-time.

### 3.2.3 Sandfly microhabitat distribution

3.2.3.1 Comparison between catches in the forest and in open areas: Captures inside houses were characterized by the following: (1) similar sandfly composition in both forest and open areas; (2) a predominance of *Lu. evansi* in both areas (87.8% and 80.45% of the total captures, respectively); (3) significantly higher density of flies (almost twice) in houses situated in open areas than those in forested areas ($F= 17.61$; $p < 0.0001$; $df= 1$), April, August and October values being the major sources for this difference ($F= 79.77$; $p < 0.0001$; $df= 9$), (Figure 3.5); (4) higher sandfly activity in porch areas (almost three times) than inside bedrooms. In other words, the number of flies inside sleeping areas (bedrooms) were comparatively lower than those outside bedrooms ($F= 13.08$; $p < 0.0006$; $df= 1$) the April figures being the main source of the disparity (Figure 3.6); and (5) total predominance of *Lu. evansi* (98%) inside the bedrooms, the activity of other sandfly species being almost negligible.

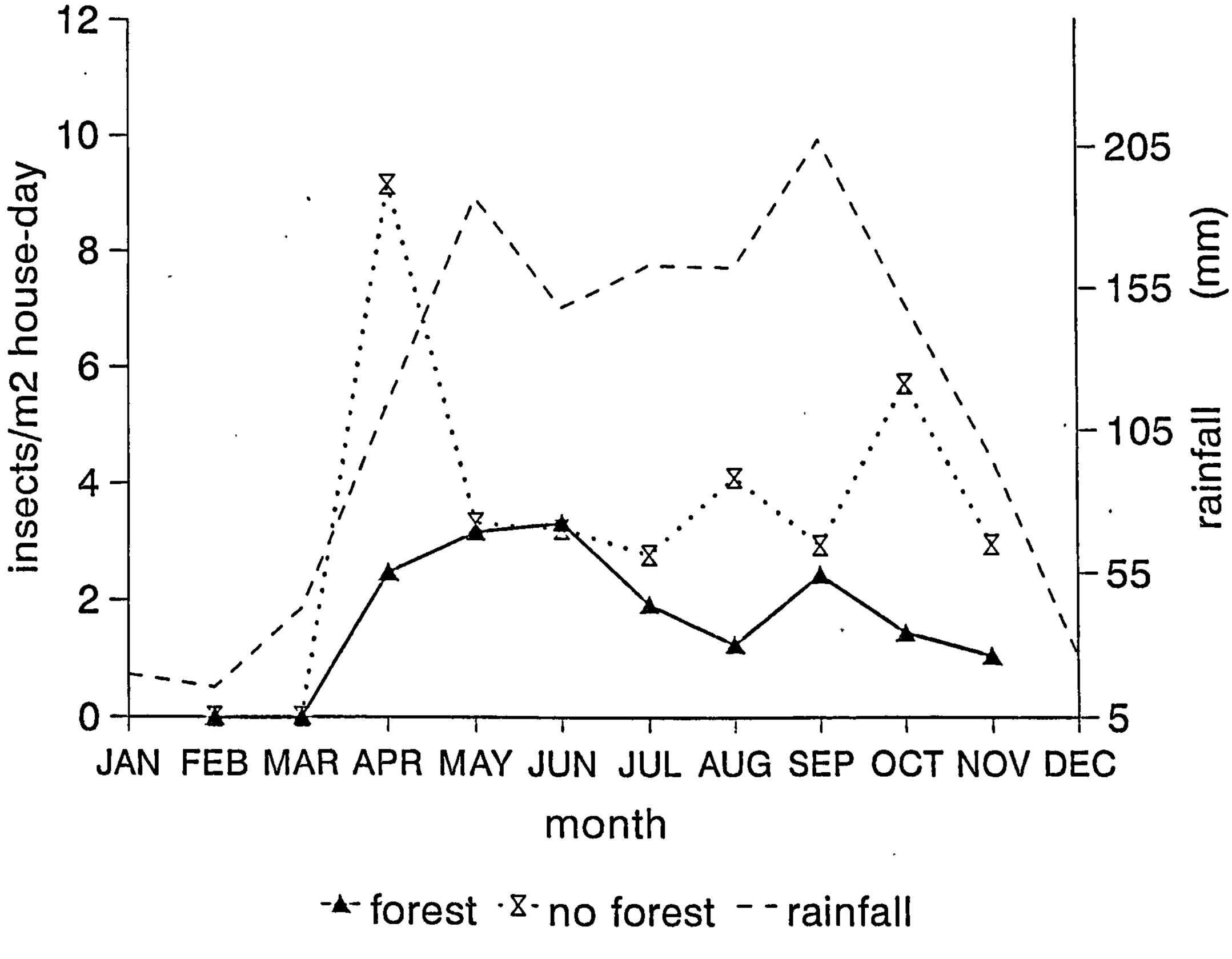


**Figure 3.5** Comparison of *Lu. evansi* sticky trap catches with respect to area (forest/open) of capture.

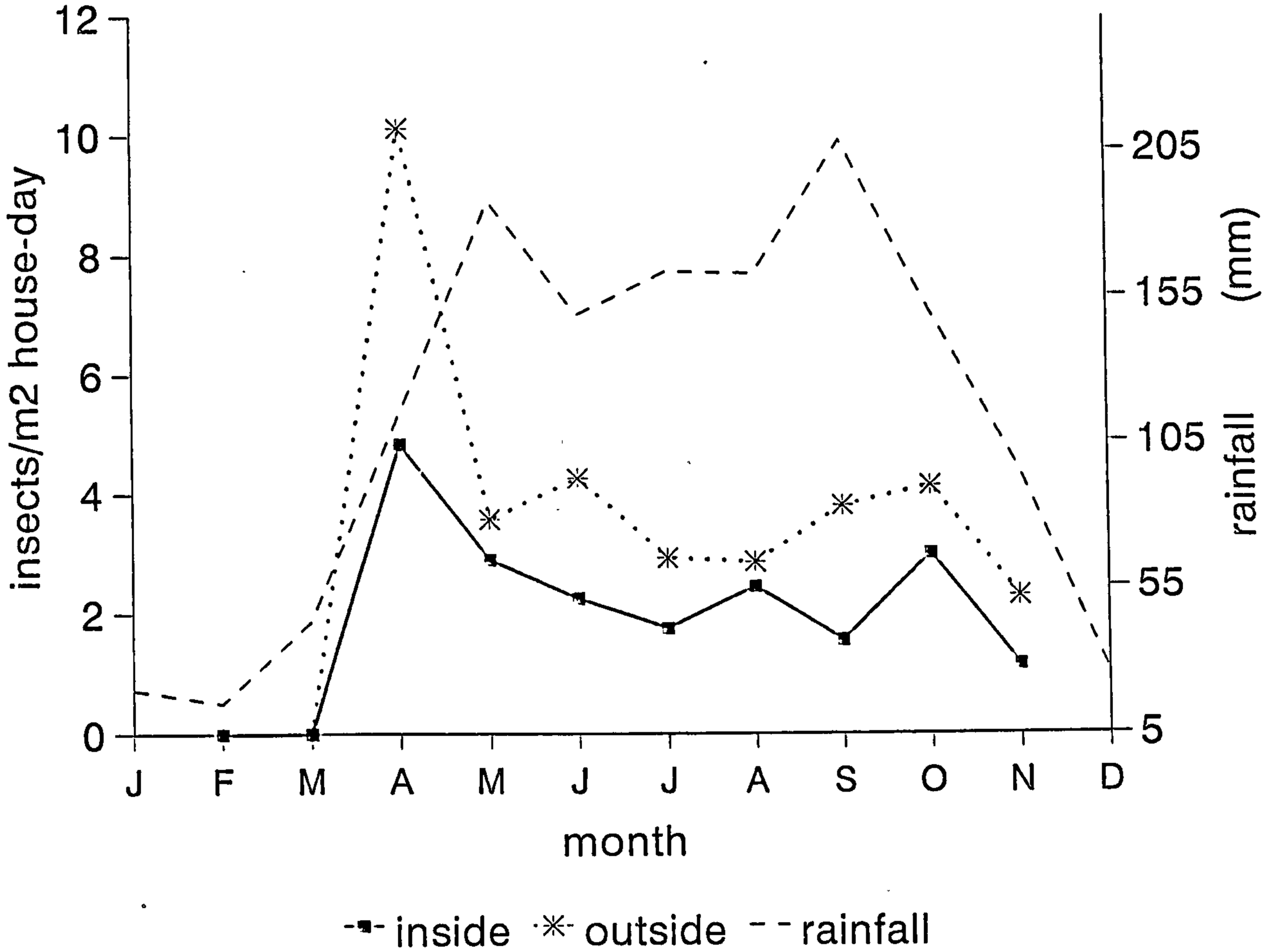


**Figure 3.6** Comparison of *Lu. evansi* sticky trap catches with respect to position (inside or outside) the bedrooms.

Overall, sex ratios of sticky-trap catches were male biased, at least during the first three months and through August. However, when the analysis was segregated by area, it became clear that males caught in houses situated near the forest area were the main source for this difference. Interestingly, one of the largest densities of males were concomitant with the beginning of sandfly activity in April while a secondary peak was in August, immediately prior to the second main sandfly peak.

3.3.3.2 Vertical distribution: Overall, no statistically significant differences were found between catches at different heights above the ground in either the open or forest setting ($F = 2.50$; $p > 0.1$; $df = 1$). However, a rough observation indicated that during a very windy August (winds greater than 0.8 m/sec) a higher (though not significantly different) number of flies was collected in traps situated in the lower position, in both environments (Figure 3.7).

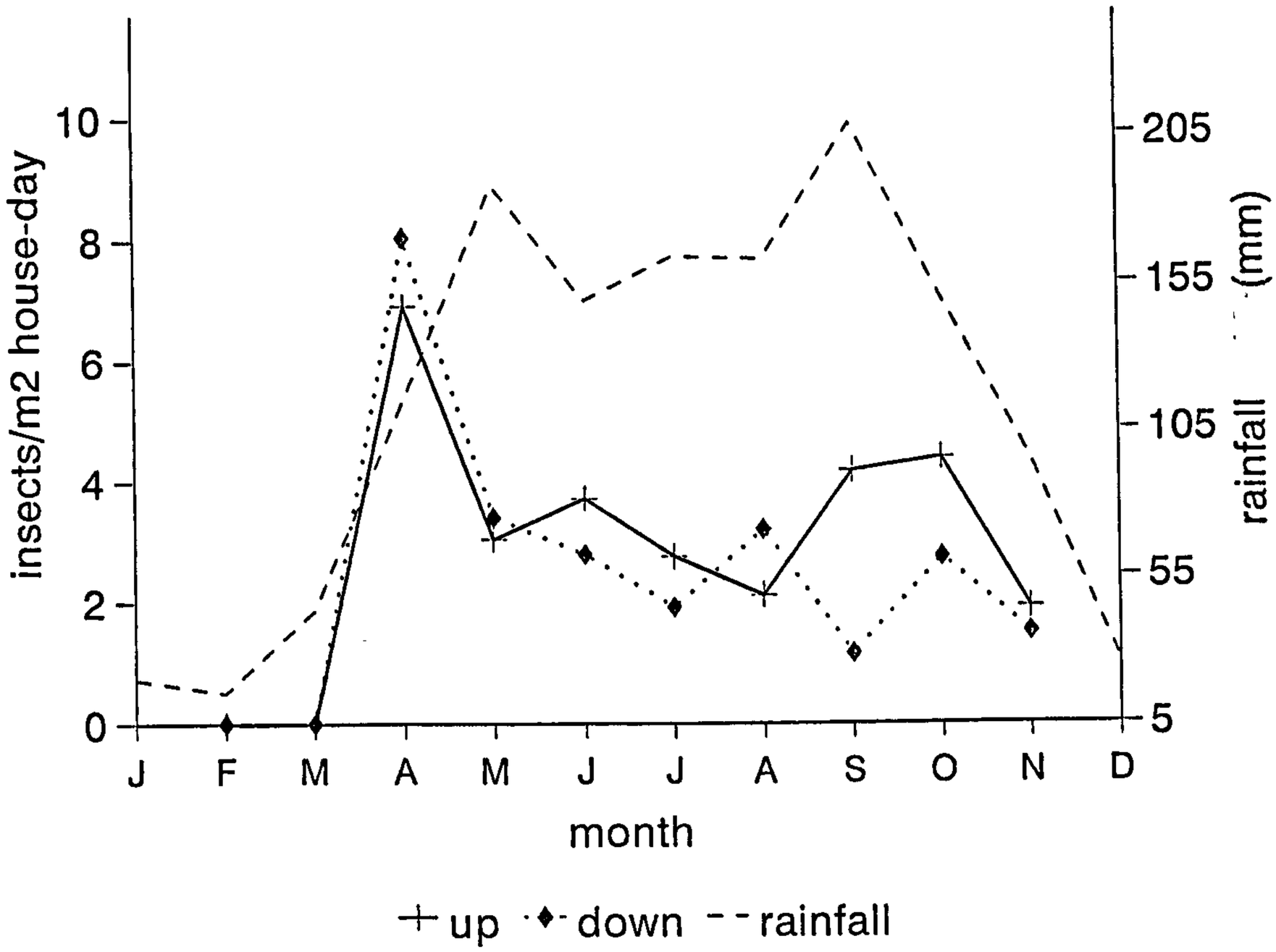


**Figure 3.7** Comparison of the number of catches of flies trapped in sticky traps inside the houses in two positions 0.1-0.15m and 1.8-2.0m above ground.

3.3.4 Daily activity pattern

Despite efforts to develop a longitudinal sampling programme with human bait, complete sets of data were only obtained for four months (April, August, October and November 1993). Because of the paucity of this information, no clear seasonal changes could be detected. However, though cautiously interpreted, results were reliable enough for determining with a high degree of confidence, the nightly biting cycle and man biting rates of *Lu. evansi*, the most abundant species (89.1%; n= 1,261). These results will be analysed and discussed in detail later, in the following chapter on host-vector contact.

Not surprisingly, results of the two-hour catches in Shannon traps showed that the rank in abundance was *Lu. evansi*, *Lu. cayennensis*, *Lu. trinidadensis* and *Lu. gomezi*. The first species accounted for 99.5% of the total wet season catches, while the presence of other species to *Lu. evansi* was almost negligible during the dry period. Besides the significant differences in the number of flies between the two periods (Figure 3.8), it was noted that the beginning of the nightly activity of *Lu. evansi* varied according to the season. During the rainy season, sandfly activity began relatively earlier, at approx. 19:15h, than it did during

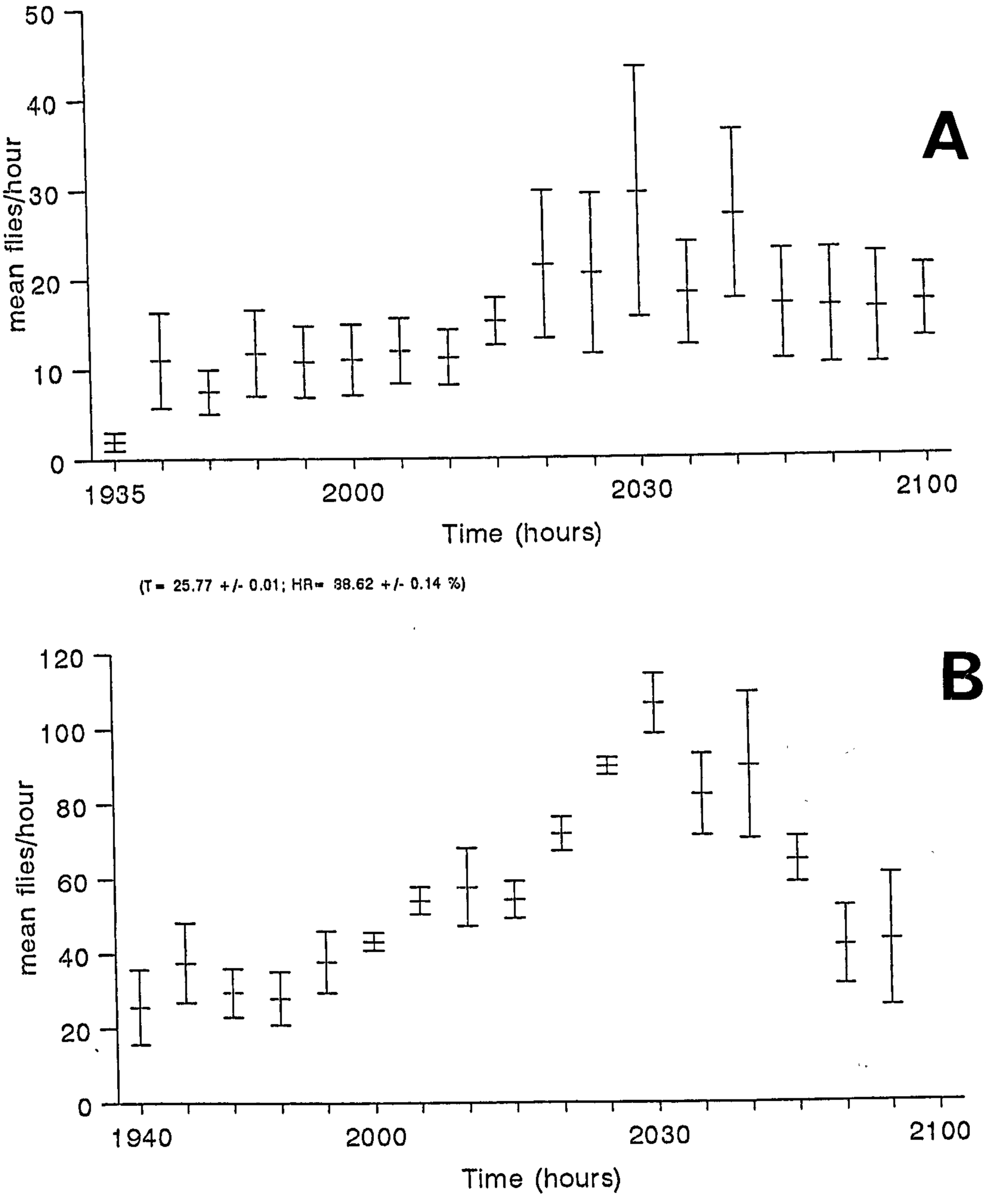


**Figure 3.8** Comparison of the number of sandfly catches using Shannon trap during (A) dry and (B) rainy season in El Contento. Bars indicate standard errors

the dry period which usually started after 19:35h. In both cases, the sandfly activity peak was recorded at 20:30h which coincides very well with the first peak of the night observed in the intra domiciliary human-bait catches.

## 3.4 Discussion

From Mexico to northern Argentina, American visceral leishmaniasis has been traditionally associated with regions of tropical dry forest. *Lutzomyia longipalpis* is usually the most abundant species (over 80%) in several of the AVL foci found throughout this geographical range; eg. Honduras (Navin et al., 1985), Costa Rica (Zeledón et al., 1984), El Salvador (Rosabal & Trejos, 1965), Colombia (Morrison 1994); Venezuela (Moreno & Oviedo, 1995) and Brazil (Sherlock & Miranda, 1993; J.Vexenat, pers. comm.). Nevertheless, it did not occur in our of study area, instead *Lu. evansi* was the predominant species in all sampled environments.

The sandfly faunal composition in the SAS area and neighbouring localities appears strikingly similar to that recently studied by (ID Vélez, per. comm.) and Gallego &Vélez (1995) in other AVL municipalities in the Caribbean Coast of Colombia. Although fragmentary, the available evidence indicate that the distribution of visceral leishmaniasis cases correspond to the areas where *Lu. evansi* has been found. Interestingly, the composition and structure of the sandfly fauna in Los Pajonales, an Andean AVL focus in Venezuela (Moreno & Oviedo, 1995) is almost identical to that recorded in our study. All of these records are consistent with the hypothesis that AVL areas in northern Colombia (probably including some Venezuelan areas), have *Lu. evansi* as the main vector of the disease. However, the lack of both historical and up-dated information on these recently discovered foci precludes any comment on the biological and environmental factors that might explain the displacement of *Lu. longipalpis* by *Lu. evansi*. As the extent of new studies on these AVL areas increase, the borders of these atypical AVL foci might be delineated more fully.

*Lu. evansi* was the most abundant sandfly present in San Andrés de Sotavento area where AVL transmission occurs. The predominance of *Lu. evansi* indicates the success of this sandfly species in colonizing domestic areas, even in places such as Vidales, Balsal and Gardenias which had experienced dramatic environmental changes (eg. clearing and burning) due to human practices.

In the case of other sandfly species, based on either their low and irregular seasonal patterns or natural history their involvement in AVL transmission in SAS can be almost disregarded. For instance, although *Lu. panamensis* and *Lu. gomezi* were found biting humans in intra-domiciliary settings, their abundance was always very low. Nevertheless, it is important to remember that though less common in SAS these two species are known to

transmit American cutaneous leishmaniasis elsewhere (Christensen & Herrer, 1973; 1980; Young & Duncan, 1994). On the other hand, *Lu. cayennensis* and *Lu. trinidadensis*, either the second or third most abundant sandflies associated with *Lu. evansi* in SAS, besides exhibiting irregular longitudinal abundance patterns their natural histories point to them as reptile feeders. This fact has been recorded (though not conclusively demonstrated) from Mexico to northern South America (Christensen et al., 1983; Young & Duncan, 1994).

The seasonal pattern of intra domiciliary sandflies found during our studies in San Andrés de Sotavento differs from that of Travi et al. (in press). The latter authors recorded highest sandfly activity during the period May and July in 1991. Since the climatological pattern in the dry Neotropics is unpredictable, we consider that this variation is not rare. According to Christensen et al. (1983) changes in the rainfall pattern are the most likely factors affecting the seasonal pattern of Neotropical sandflies. In Colombia, meteorological data compiled by the HIMAT (the national climatological institute) showed a great variation on the regime of rains between September 1991 and August 1992. As a consequence, an exceptional drought was experienced in the Caribbean coast as well as other areas of Colombia. In the particular case of SAS, the rainy period was delayed and when it was finally came, the level of rainfall was significantly lower than previous years. According to this, one would expect sandfly populations to vary from one year to the next, as occurred during our study period. However, the most remarkable common point in these studies is the detection of the post rains bimodal abundance pattern in *Lu. evansi*. This must be taken into account when developing any control strategy directed against the vector.

An important issue is the sudden increase in sandfly numbers observed in April. As a consequence of the drought, sandfly populations dropped to almost zero between December 1992 and March 1993, *Lu. evansi* being almost the only species detectable, though in very low numbers, and mainly in vegetation patches. Although it is plausible that these patches represented "refuges" to the sandfly populations, it is hard to explain how such a diminished population could generate a high, almost spontaneous, number increase just 10-20 days after the first rains. One possible explanation for this event, might be in the existence of short intrinsic developmental periods in the local sandfly population. However, laboratory data on the biology of *Lu. evansi*, the most abundant species in the area, indicates that the minimum length of its development is 35 days (at 32°C) (Mirsa, 1953; present study). Although we have

no homologous information of this range in nature, it is unlikely, at least in *Lu. evansi*, that such increase as that observed could be generated by a rapid augment in reproduction. A second explanation, relies on the occurrence of survival strategies such as dormancy in the sandfly population. Although this phenomenon has been reported in other sandfly species (Chaniotis & Anderson, 1968) and is suspected in *Lu. longipalpis* (Morrison, 1994) more detailed studies are required to detect any intra-specific variation in the nature of this dormancy (Danks, 1987; p.19-45). An observation which might lend support to the idea of diapause in *Lu. evansi* is the presence of at least two adult "forms" of different size. Small *Lu. evansi* were found all the year round, being totally dominant in April immediately after the first rains. Bigger individuals were found in July. Although unfortunately our observations were not quantified, we consider that the small size might correspond to individuals emerging from a dormant population. Loss of 12% of body weight has been reported as a direct consequence of dormancy in the sawfly larvae *Trichiocampus populi* (Sakagami & Tanno, 1979 cited by Danks, 1987 p.39) and in adult *Culex pipiens* mosquitoes (Mayika & Taguchi, 1982) which may lose over 44% of their body weight. On the other hand, we are aware that difference in size, per se, might be not a clear indicator of dormancy but a simple seasonal polymorphism as observed in *Lu. longipalpis* in Bolivia (Bonnefoy et al. 1986).

During the dry season, characteristically from November to April, some trees lose part of their foliage allowing the sunlight to desiccate the forest floor and produce "broken soil" or the typical cracks of drought. Since sandfly abundance in the area was correlated with rainfall, it was expected that abundance would vary through the summer, the adult of most species were absent in the dry season. Given that most Neotropical sandflies appear to be susceptible to desiccation it is reasonable to assume that finding suitable resting/breeding places represents the most limiting parameter for sandfly survival during summer period. For species such as *Lu. panamensis* and *Lu. gomezi*, which have been reported to prefer leaf litter and shrubs or human dwellings day-time resting sites (Hanson, 1968; Christensen et al., 1983) the dryness accompanied by changes in the amount of organic matter in the soil might explain their irregular seasonal pattern. For other species, such as *Lu. trinidadensis* and probably *Lu. evansi* which coexist as the main "dwellers" of arboreal places, their preferred microhabitats might be less susceptible to seasonal changes. It is tempting to postulate that the degree of preference exhibited by some flies for some tree species (eg. *G. sepium*, *C. pentandra* and above all for *Gu. ulmifolia*) might indicate a degree of sandfly dependence which in turn

determines the survival of some species. This dependence is most noticeable during the dry season, when finding suitable resting places becomes harder. During the wet period, humidity is much higher and there is a surplus of suitable resting places.

A final observation on resting places is that in SAS rocky buildings or limestone caves are not as obvious as they are where *Lu. longipalpis* is present. *Lu. longipalpis* has a propensity to rest in these kinds of rocky structures (cf. Morrison, 1994; Ferro et al., 1995; pers. obs. in northeast Colombia). This important observation, might yield some insight into the factors differentiating habitats between the two AVL known vectors *Lu. evansi* and *Lu. longipalpis*.

# CHAPTER 4

# HOST-VECTOR CONTACT

## 4.1 Background

Transmission cycles of arthropod-borne diseases are regulated by both extrinsic and intrinsic factors. Whether or not a vector will come in contact with suitable vertebrate reservoir/hosts of parasites is an important extrinsic factor (WHO, 1972), while a vector's predilection for a host is an intrinsic factor. Both affect the vectorial competence of a vector, ie. the overall ability of a species in a given location at a specific time to transmit a pathogen.

Studies on host attraction and feeding behaviour in sandflies indicate that both New and Old World species display varying degrees of host preference but to a great extent all are opportunistic feeders. For instance, *P. papatasi* is considered a highly anthropophilic species by several workers in India (Namita et al., 1991; Mukhopadhyay et al., 1987; Dhanda & Gill, 1982) but it has been also observed biting several animal species, in the same country (Namita et al., 1991; Dhanda & Gill, 1982) and Egypt (El Sawaf et al., 1989). In Sri Lanka, *P. argentipes*, although predominantly zoophilic in lowland areas prefers human beings in the highlands (Lane et al., 1990). Other studies reveal that the blood feeding preferences of some Ethiopian (Foster et al., 1972) and Kenyan (Mutinga et al., 1986; Johnson et al., 1993) sandfly species are affected, among other factors, by host availability and size. Studies on attractiveness of sylvatic and domestic animals to *Lutzomyia* species in the New World (including the main vectors of leishmaniases) have shown that most of them are attracted to a wide number of hosts and feed upon them in an opportunistic way (Christensen & Herrer, 1973; 1980; Tesh et al., 1971, 1972; Quinnell et al., 1992; Morrison et al., 1993b). By contrast, very few species have distinct host preference patterns (eg. small rodents in *Lu. flaviscutellata* (Shaw & Lainson, 1968), bats in *Lu. vespertilionis* and lizards in *Lu. trinidadensis* (Young, 1979)). Thus, the eclectic behaviour of sandfly species is a predominant

and important event in zoonotic leishmaniasis cycles since intensity of transmission of *Leishmania* parasites is determined, not only by the total number of bites on humans but primarily by the probability of vector feeding on the vertebrate host population, which usually represents its major source of blood-meals (Bray, 1982). Furthermore, the reservoir usually keeps a high infection rate of the parasite (Dye, 1994).

Historically, the transmission cycle of *Leishmania chagasi* by *Lu. longipalpis* involves dogs and foxes as intermediate hosts (Lainson et al., 1985). However, in San Andrés de Sotavento, where *Lu. evansi* is the known vector of *L. chagasi* (Travi et al., 1990; Travi et al., in press), an extensive search for reservoirs in the area showed that, in addition to dogs, the opossum *Didelphis marsupialis*, might play a role as a natural reservoir of *L. chagasi* (Travi et al., 1994). Thus, the available evidence suggests that in the San Andrés focus *Lu. evansi* is the vector of *L. chagasi* with dogs and *D. marsupialis* as the zoonotic reservoirs of the parasite.

Throughout its geographic range *Lu. evansi* is assumed to be largely an anthropophilic species (Young & Duncan, 1994); however, few workers have actually demonstrated this. Furthermore, very often the assumptions of "anthropophilic" behaviour are based on anecdotal data rather than on controlled experiments (cf. Zeledón et al. (1984); Feliciangeli et al. (1992)). In Colombia, Travi et al. (in press) found that *Lu. evansi* is the main sandfly species collected on human bait in both intra-and extra-domiciliary areas in the San Andrés VL focus. They also noted that, using Malaise traps, pigs attracted relatively more *Lu. evansi* than donkeys. In addition, it was observed that *Lu. evansi* fed on a wide variety of either anaesthetized or restrained baits (dogs, ducks, chickens, rats and opossums). However, because of limitations in their experimental design they were unable to draw any conclusion on host preference. The anthropophilic behaviour of *Lu. evansi*, therefore, still remains unclear.

The present chapter summarizes a series of controlled experiments[1] planned to determine precisely the anthropophilic behaviour of *Lu. evansi* and, in doing so, to determine

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[1] Part of the results of these experiments have been the subject of two posters, one presented during the meeting of the American Society of Hygiene and Tropical Medicine (ASHMT, Cincinnati, 1993) and, the second in the II International Symposium of Phlebotomine Sandflies (ISOPS II, Merida, 1995), respectively. Also two scientific articles were derived from them (cf. Montoya & Lane, 1996a and 1996b). The manuscripts in their original format, as well as the abstracts of the posters are presented in the appendixes 4.1-4.4.

and compare its rate of contact with domestic and wild animals which might be potential sources of blood/parasites.

## 4.2 Methods

### 4.2.1 Experiment 1: Relative host preference for reservoirs and humans

This subject was measured by presenting a choice of three baits and a blank control in specially designed cone-traps (Montoya-Lerma & Lane, 1996).The device consists of a rectangular tent (2.0m width x 1.5m height x 2.0m long) made of light fabric with netting windows on two sides (Figure 4.1). The unit had a waterproof, polyvinyl floor; two nylon-zippered entrances for the collector and two inward pointing cones with modified entry points which allow easy access for flies, yet precludes their escape. The total unit is supported

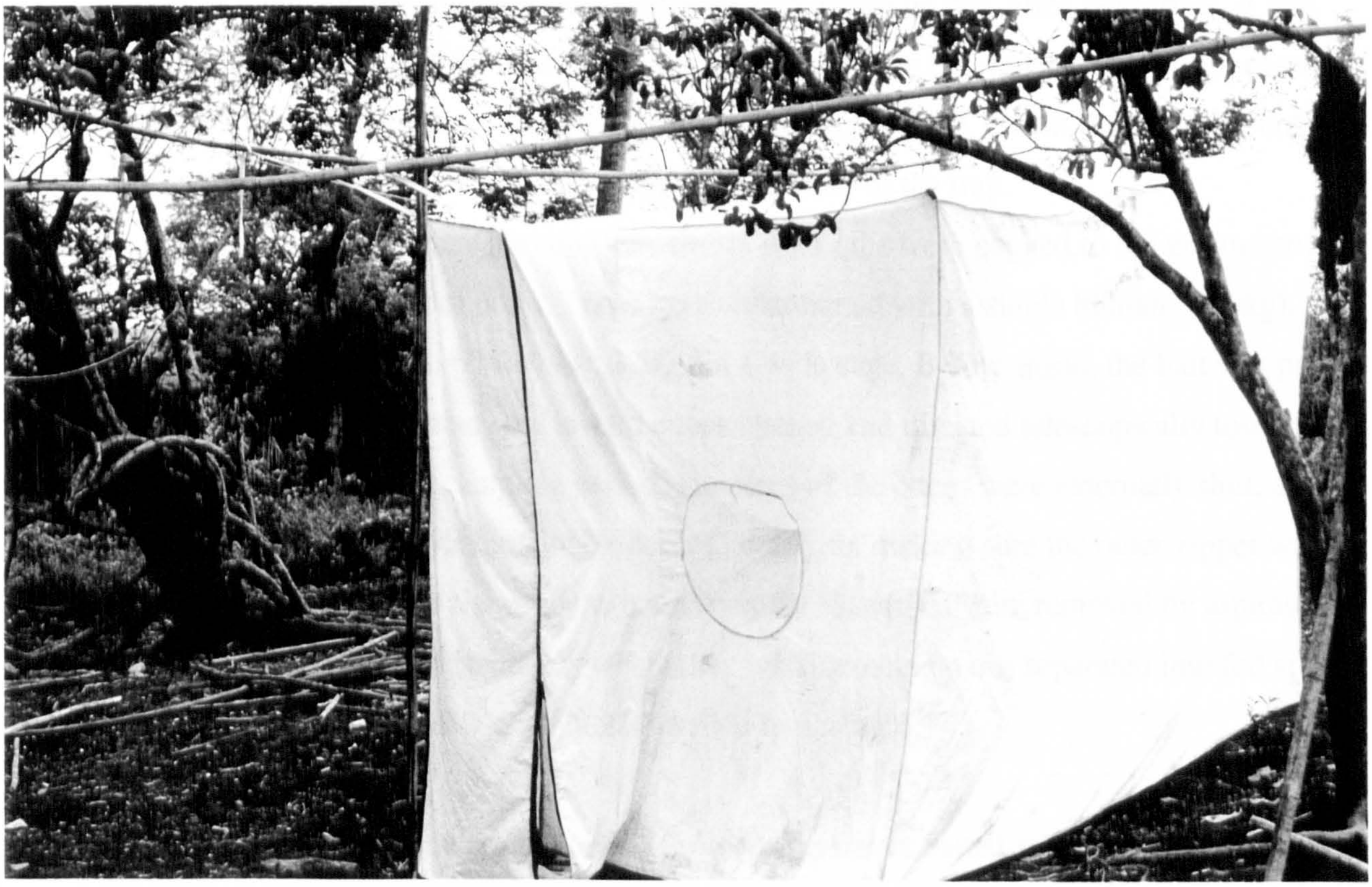


**Figure 4.1** The cone-trap in operation

externally by metal rings from eight wooden poles in cemented containers. All materials for the unit were purchased locally. The chamber, the key section of the trap, consists of two sides and the roof made from three rectangular pieces of Dacron (2.0 x 2.0m each). Two

rectangular windows were made by cutting a hole (75cm x 30cm ) one in the top and other in the bottom half of each side, then covered with sandfly proof, nylon netting. The two remaining sides were made from the same fabric, 1.5 x 2.0m and a circular hole 30cm diameter was cut in the centre of each panel. Two fabric cones (80 cm long x 30cm OD x 15mm ID) were sewn in the holes. A plastic film case was secured at the top of each cone with rubber bands. One side of the case was modified by cutting a hole with a knife. A piece of heavy duty plastic polyvinyl (2.0 x 20m) was used as the floor. All parts, except the front were sewn together with nylon thread to form a cuboid tent. A double-sided zipper was sewn in an L shape down one of the vertical edges and along the front of the floor of the trap. Externally, reinforced leather tabs holding metal rings were sewn at each corner of the trap. Two additional tabs were sewn halfway along the edges of the floor and roof. All joins were sewn that the cut edges were facing outwards ensuring that the inside of the seam was smooth. Originally, the trap was designed without a vestibule but, after field trials, it was noted that some flies escaped while the collector was entering the trap through the zippered entrance. To overcome this, a small rectangular section (0.5m wide, 1.20m high x 0.5m long) with an additional zipped entry was attached to the front of the trap.

To set up and operate the cone trap, the six floor tabs were pegged to the ground and the roof tabs tied to external poles. Traps were either baited with a single human (c. 70kg), a dog (20kg) or an opossum (*Didelphis*, 2.5kg) in a wide cage. Before dusk, the bait was put in the middle of the chamber, the inward cones opened and directed telescopically towards it. For inspection, after a sampling period, the cases of the cones were externally shut, and collector entered the trap via the "double doored" entrance, making sure the outer zipper was closed before opening the zipper to the main chamber. Sandflies were removed by aspirator and transported to the field laboratory where they were sexed, females separated into fed and unfed, and all identified after dissection and slide-mounting.

4.2.1.1 Determining host attractiveness: The cone-traps were baited with a single human (c. 70kg), dog (20kg) or opossum (2,5kg). Figure 4.2 indicates the arrangement of the four cone-traps in field conditions. Each trapping position was five metres from the wall of a house. Each host, together with its trap (to avoid bias from residual odour), was rotated to the next trapping position the following night. Thus, after four consecutive nights each bait or the control had been presented at each trapping position to give four replicates.

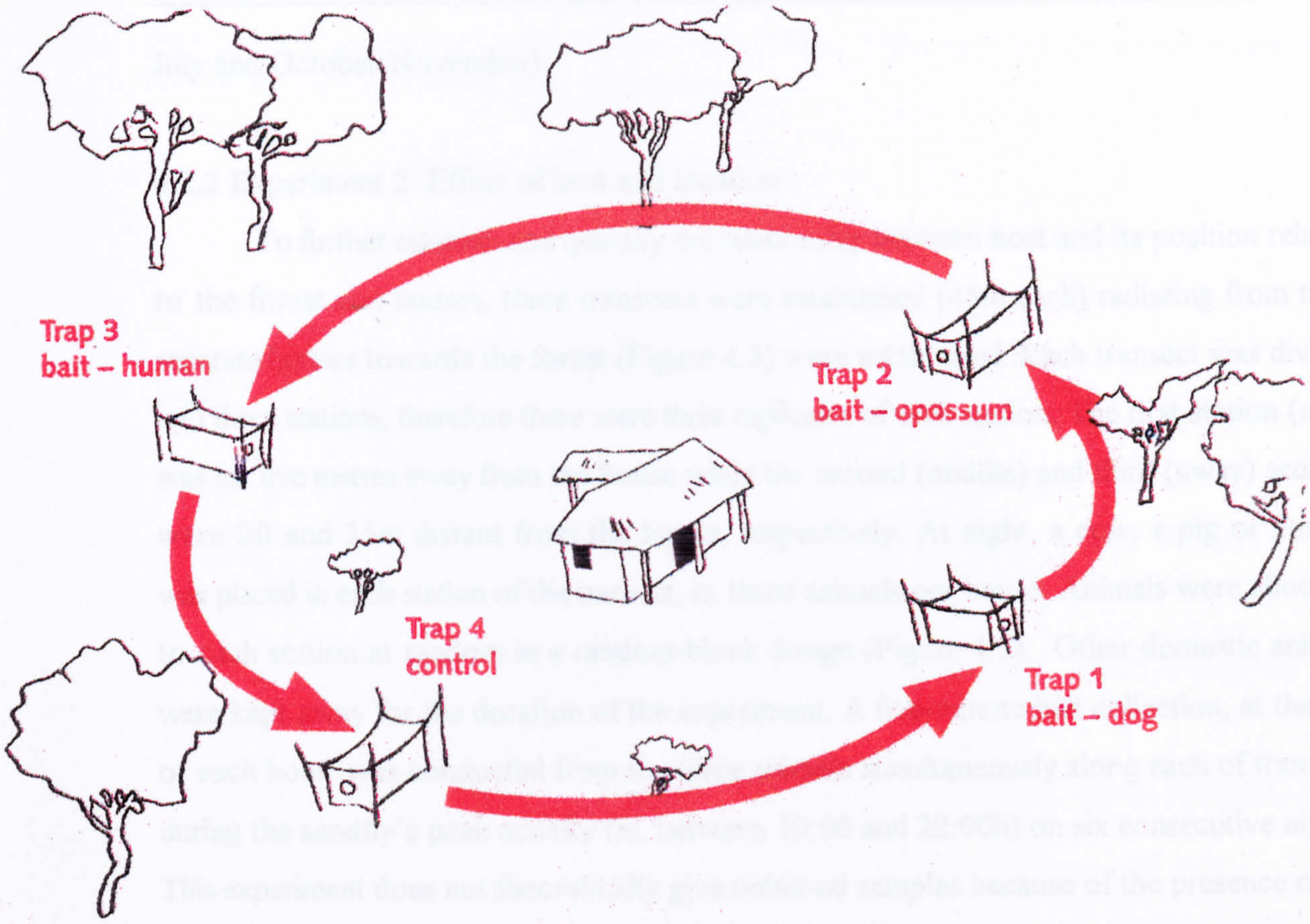



**Figure 4.2** Arrangement of cone traps and their baits; each was rotated daily as indicated by arrows.

4.2.1.2 House location: To determine the effect of proximity to the "forest", this four-nightly procedure was carried out around two houses in the village of El Contento, one house with the "forest" edge within five metres, the other house with the "forest" edge more than 10 metres away, termed "forest" and "open area", respectively. The houses were matched for the domestic animal composition and the human: domestic animal ratio. These factors remained relatively constant throughout the study period.

In summary, the total experiment involved 56 trap-nights in which the effect of bait, proximity to forest and season could be distinguished from the potentially confounding factors of site and day to day variation.

4.2.1.3 Seasonal host preferences: To detect any seasonal change in host preference, the experiment (eight trap-nights) was repeated seven times throughout the year to cover periods in the rainy season (March-April and August-September) as well as the drier seasons (June-

July and October-November).

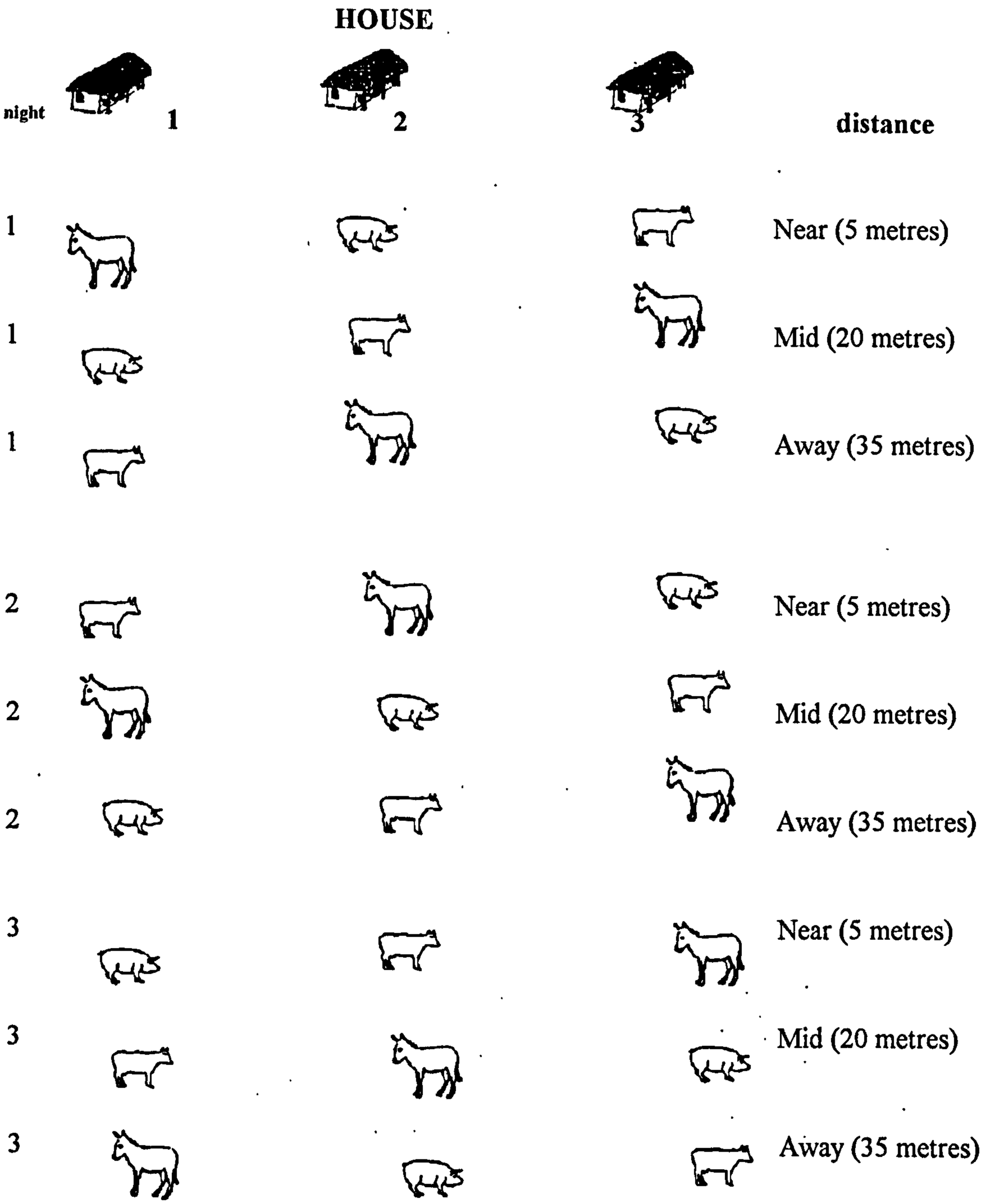
4.2.2 Experiment 2: Effect of host and location

To further establish and quantify the relationship between host and its position relative to the forest and houses, three transects were established (45m each) radiating from three separate houses towards the forest (Figure 4.3) were established. Each transect was divided into three stations; therefore there were three replicates of each station. The first station (near) was ca. five metres away from the house while the second (middle) and third (away) sectors, were 20 and 35m distant from the house, respectively. At night, a cow, a pig or donkey was placed in each station of the transect, ie. three animals per house. Animals were allocated to each station at random in a random-block design (Figure 4.3). Other domestic animals were kept away for the duration of the experiment. A five-minute bait collection, at the end of each hour, was conducted from the three animals simultaneously along each of transects during the sandfly's peak activity (ie. between 19:00 and 22:00h) on six consecutive nights. This experiment does not theoretically give unbiased samples because of the presence of the human collector. However, since the animals were only sampled for 5 min/hour and it usually takes more than five minutes for sandflies to come to a human bait the effect of collector is probably minimal. The experiment was undertaken in El Contento, between end of July and beginning of August 1993.

4.2.3 Experiment 3: Sandfly movement in relation to blood-feeding

To investigate the movement of blood-fed sandflies in and around houses and their natural host preference, a sampling procedure was developed combining sticky traps (night) and direct search (daylight) captures. Four transects (A,B,C,D) radiating from a house towards the forest in a semi circle were established, along which were four quadrats (20 x 15m) at fixed distances to 100m (A1, A2, .....D4) making 16 quadrats in all. In addition, each quadrat was subdivided into 20 sampling points. Thus, the experimental area covered places with different classes of vegetation where the main domestic animals (cow, pig, donkey and chickens) spend the night (Figure 4.4). At dusk, 20 oiled sticky traps (ST) were set in each quadrat of two transects (making a total of 160 ST's per night). Further sets of sticky traps were distributed inside the house (intradomicile) and in a patch of forest beyond the transects to act as a general reference point for sandfly abundance. Traps were left overnight and the following morning, simultaneously with the inspection of the traps, 160 sampling points

**Figure 4.3**: Random block design (Experiment 2). Each night, the baits from each house were rotated of position respect from the house.



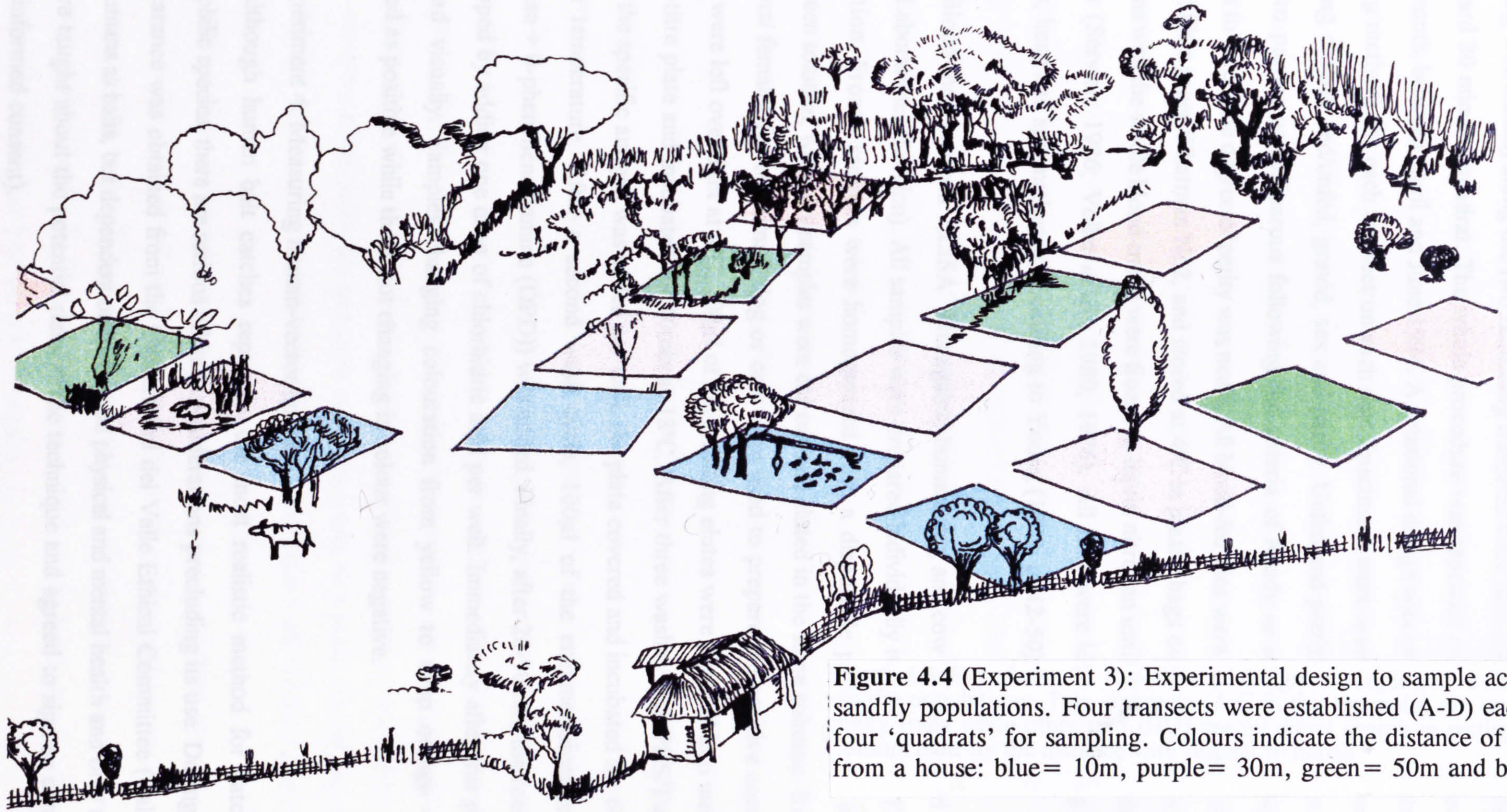


**Figure 4.4** (Experiment 3): Experimental design to sample active and resting sandfly populations. Four transects were established (A-D) each containing four 'quadrats' for sampling. Colours indicate the distance of the quadrats from a house: blue= 10m, purple= 30m, green= 50m and beige= 70m.

(including tree trunks) along the two remaining transects were searched for resting flies for a standard 20 minutes/quadrat. The whole procedure was repeated over eight consecutive nights/month, between April and June 1994. A rotational design was established, alternating sampling methods for each transect on each day. Specimens were sorted to species and the following criteria: fed/unfed, gravid, sex and parity. Unfed and partially fed females were sorted to parous or nulliparous following the criteria of Ready et al. (1984) though no attempts to determine degree of parity was made. All blood-fed flies were either smeared onto pieces of filter paper Whatman No2, and stored at 4°C in plastic bags over silica gel, or their abdomens with the whole blood-meals were frozen in liquid nitrogen until blood-meal ELISA analysis (Service, 1986; Voller et al., 1980; 1976). All flies were identified using body carcases, head and spermathecae, according to Young (1979, p. 42-50).

4.2.3.1 Blood-meal analysis: ELISA kits against human, dog and cow were obtained from Bryllert Laboratories (London). All samples were prepared individually in 0.5ml PBS/Tween 20 solution. Frozen samples were homogenized with a disposable plastic pestle in the PBS/Tween solution, while dry samples were cut out and eluted in the same solution. Reared *Lu. evansi* females fed on human, dog or cow were used to prepare the positive controls. Samples were left overnight at 4°C. 100µl of the resulting elutes were pipetted into wells of a micro-titre plate and incubated for 60min at 18°C. After three washes with PBS/Tween, 100µl of the specific antigen was added per well, the plate covered and incubated for 60min at room temperature. After a second wash cycle, 100µl of the enzyme/substrate (ie. Peroxidase + 0-phenyllenediamine (OPD)) was added. Finally, after 20-30min, the reaction was stopped by adding one drop of chlorhidric acid per well. Immediately after this plates were read visually. Samples changing colouration from yellow to deep orange were considered as positive while those not changing in colour were negative.

4.2.4 Experiment 4: Measuring human-vector contact

Although human bait catches represent the most realistic method for catching anthropophilic species, there are serious ethical considerations precluding its use. During this study clearance was obtained from the Universidad del Valle Ethical Committee (Cali) to employ humans as baits, but dependent on their good physical and mental health and only after they were taught about the potential risks of the technique and agreed to sign an consent form (ie. informed consent).

The main method of estimating human/ *Lu. evansi* contact was the landing count technique. Briefly, one person was allocated per house with clothed legs and arms to catch all flies landing on him with an aspirator. Human bait trapping covered both wet and dry seasons. When possible, human baiting was done for eight consecutive nights/month, between 19:00 and 06:00h for 50mins/hour. During each 50min period, all captured sandflies were blown into a Nalgene® plastered holding pot. Biases in the human bait catches were minimized by:

* Taking into account people's behaviour: since people usually remain in the porches until 20:00h, catches were initiated in the porches area and then at bed time, they were switched to inside bedrooms. It is important to note that structurally and physically there is no clear difference between the place where the people spend the night (bedroom) and the living room.
* Shifting time and rotation of catchers. People worked in two shifts: 19:00- 24:00 and 24:00 to 05:30h. In order to reduce effects due to some catchers being more attractive than others (Khan et al., 1971; Lane et al., 1990) or having better skills for catching flies, the collectors were rotated between houses each day.
* Training and inspection. Catchers were trained to pick up all sandflies landing on themselves and to keep the hourly captures separately. They were regularly inspected to correct possible errors.
* Covering torches with red cellophane. The goal of this was prevent the possible attraction effect of torchlight.
* matching houses (see section 3.2.2)..In some experiments, biting catches were replicated using matched houses. Unfortunately, this ideal was not always possible. Considerable difficulty was experienced getting permission to work inside houses. Unwillingness to cooperate was explained sometimes as the disruption to privacy (houses usually consist of a single bedroom where almost all the family slept) or sometimes because of mis-information about the objective of the study (some people were suspicious or scared). At the beginning of the study eight houses were sampled; however, complete data were obtained from four houses only.

Hourly set of sandfly captures were dissected next morning to establish identification, parous rate and to detect natural infection (Chapter 7). In addition, in some instances, samples from the hungry flies were allowed to feed on hamsters to determine the *Lu. evansi* gonadotrophic cycle (Chapter 6). The captures were carried out in April, August, October and November, in at least four houses of El Contento.

4.2.5. Statistical analysis

As mentioned above, the focus of the cone-trap experiments was to compare the relative attractiveness of baits in different habitats. During a pilot study (data not shown) it was found that the distribution of flies between nights and between the different baits, was not normal but approximated a Poisson distribution. A Kruskal-Wallis analysis of variance (ANOVA) for a Latin Square design (Fowler & Cohen, 1990 p.201) was therefore used to calculate the most likely marker (factor) associated with both attraction (flies found in a trap) and feeding behaviour (flies blood-fed in a trap). Once the markers were selected the expected numbers of unfed and engorged females were estimated by separate linear regression models assuming un-baited conditions. Subsequently, in order to control for other factors (season, forest, night, position), the expected values were used as co-variables in a covariance analysis with one predictor factor (bait). A chi-squared analysis was used to compared the blood-feeding between baits and between habitats. Finally, the feeding index (FI) was calculated according to Kay et al. (1979):

$$FI = (Ne/Ne')/(Ef/Ef')$$

where Ne and Ne' are the number of fly blood feeds on hosts 1 and 2 respectively, and Ef and Ef' are the expected proportion of feeds on hosts 1 and 2, respectively. Because this experiment tests attraction to a bait, we have assumed that entering a trap is independent of the local abundance of the host animal in calculating the expected proportions. Thus, a FI greater than 1 indicates an increased amount of feeding on host 1 relative to host 2.

In the second set of experiments (effect of host location) the analysis was based on the model using one transect/house; three stations/area and three houses/night for a total of 54 samples. The number of individuals collected per sample was recorded and sorted into fed and unfed flies. Unfed females were dissected to determine physiological stage (ie. nulliparous/parous). Fly frequencies were log transformed and multifactorial ANOVA was chosen as a suitable method to compare the frequencies of captures, controlling for distance from houses, bait, blood feeding and parity.

Catches from the sandfly movement in relation to blood-feeding experiment (experiment 3) were transformed to log (n + 1) and analysed by ANOVA to detect any

differences between host, places and changes in abundance during sampling. The density of sandflies is presented by the back-transformed geometric means and compared by $\chi^2$ to test for the goodness of fit to the negative binomial distribution.

Finally, the information derived from the discrete human landing captures (experiment 4) was used to determine the nightly activity cycle, human biting rates (HBR) and, to some extent, the endophagic and endophilic rates (ie. the tendency of flies to feed and rest indoors, respectively). In all cases, raw data were log-transformed prior any analysis.

## 4.3 Results

A total of eight *Lutzomyia* species were identified through the experiments: *Lu. evansi*, *Lu. cayennensis*, *Lu. trinidadensis*, *Lu. rangeliana*, *Lu. gomezi*, *Lu. panamensis*, *Lu. shannoni* and *Lu. dubitans*. Their abundance varied temporally and according to sampling method. Overall the first three species were the most common, accounting for 98% of the captures. Therefore, unless otherwise stated, the analyses were centred on these three species.

### 4.3.1 Experiment 1: Host preference for reservoirs

With the exception of *Lu. dubitans*, which was absent from cone-trap captures, the seven remaining species of flies were caught in variable numbers in this type of trap. *Lu. evansi* was the most numerous in each baited-trap. There was no significant difference in species composition between catches in cone-traps and sticky traps run parallel inside houses, though there were differences in the species proportions and in seasonal patterns (data not shown).

4.3.1.1 Host attractiveness: From a total of 598 specimens caught in the cone traps during the complete experiment, 83.8% were *Lu. evansi* (females and males), therefore all data analysis is focused on this species because of its abundance and epidemiological importance. Only 14 males and three unfed females were caught in un-baited traps. In both open and forest habitats all hosts attracted significantly more *Lu. evansi* than the control (forest $\chi^2 = 23.50$; open $\chi^2 = 25.10$; df= 4; $p < 0.0001$) during the 56 trap-nights period (Table 4.1). Interestingly, females accounted for the highest value of these differences (forest $\chi^2 = 19.9$; $p = 0.0002$; open $\chi^2 = 36.6$; $p = 0.0001$; df= 3).

Analysis of data, taking out effects of night to night variation and position, revealed that there was a trend in the number of flies caught in traps with different baits. Overall, human caught more than either dog or opossum (Table 4.1; $p < 0.05$) but there was no significant difference between dog and opossum ($p > 0.05$). This host preference pattern is the same in the forest and open areas. Traps in the open habitat (Table 4.1) caught more flies than in the forest habitat (for all hosts), but this difference was not significant ($\chi^2 = 0.1062$; $p = 0.25$).

4.3.1.2 Seasonal host-preferences: Total fly numbers from the forest and open areas were pooled to detect any seasonal changes in the host preference of *Lu. evansi*. Firstly, there was a clear seasonal variation in sandfly abundance (Figure 4.5), with the majority of flies caught in July-August.

At the end of April, concomitantly with the first rains, sandfly numbers were low and there was no significant difference between baits. During the month of peak abundance, humans were greatly preferred over dogs and opossums. In August, catches began to decrease but man was still the preferred host until October when there was no clear preference, but a few more flies were caught on opossums. There were no detectable host preferences until December, when man began again to be the preferred host.

4.3.1.3 Sex ratio: In all baited-traps, the sex ratios were always biased in favour of females (Table 4.1). However, in control traps, males were relatively more abundant, and more so in the open area (38%) than in the forest (25%). There was no significant difference, in sex ratios between hosts, though in all cases, the absolute number of females was higher on human than on other baits.

4.3.1.4 Feeding behaviour: From a total of 332 host-seeking females caught in the baited cone-traps only 57% of them were blood-fed, but the proportion blood-fed showed significant differences between baits ($F = 13.27$; $df = 4$; $p < 0.0001$). A $\chi^2$ analysis of blood-feeding between pairs of baits showed that in the forest site more *Lu. evansi* females fed on humans than they did on opossums ($\chi^2 = 5.44$; $p < 0.05$) but no significant differences were found between human and dog ($\chi^2 = 0.71$; $p > 0.05$) or between dog and opossum. In contrast, in the "open area ", more flies fed on dog and opossum than on human, (although this was not statistically different between human and opossum).

**Table 4.1.** Total numbers of males and females; sex ratio (%♀♀) and percentages blood-fed females (% BF) of *Lu. evansi* collected in un-baited (control) and baited cone traps dog (D), opossum (O) and human (H) in forest and open areas. San Andrés de Sotavento (Córdoba- Colombia).

| | FOREST | | | | OPEN AREA | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **BAIT** | ♂♂ | ♀♀ | % ♀♀ | % BF | ♂♂ | ♀♀ | % ♀♀ | % BF | $\bar{x}$ % ♀♀ |
| **C** | 1 | 3 | 75 | 0 | 13 | 0 | 0 | 0 | 38 |
| **D** | 11 | 25 | 70 | 84 | 17 | 25 | 60 | 64 | 65 |
| **O** | 5 | 20 | 80 | 70 | 23 | 33 | 59 | 49 | 70 |
| **H** | 25 | 81 | 78 | 90 | 71 | 148 | 68 | 33 | 73 |
| **Total** | 42 | 129 | --- | --- | 124 | 206 | --- | --- | --- |
| $\bar{x}$ ♀♀ | 10.5 | 32.3 | 76 | 81 | 31 | 51.5 | 47 | 49 | 62 |

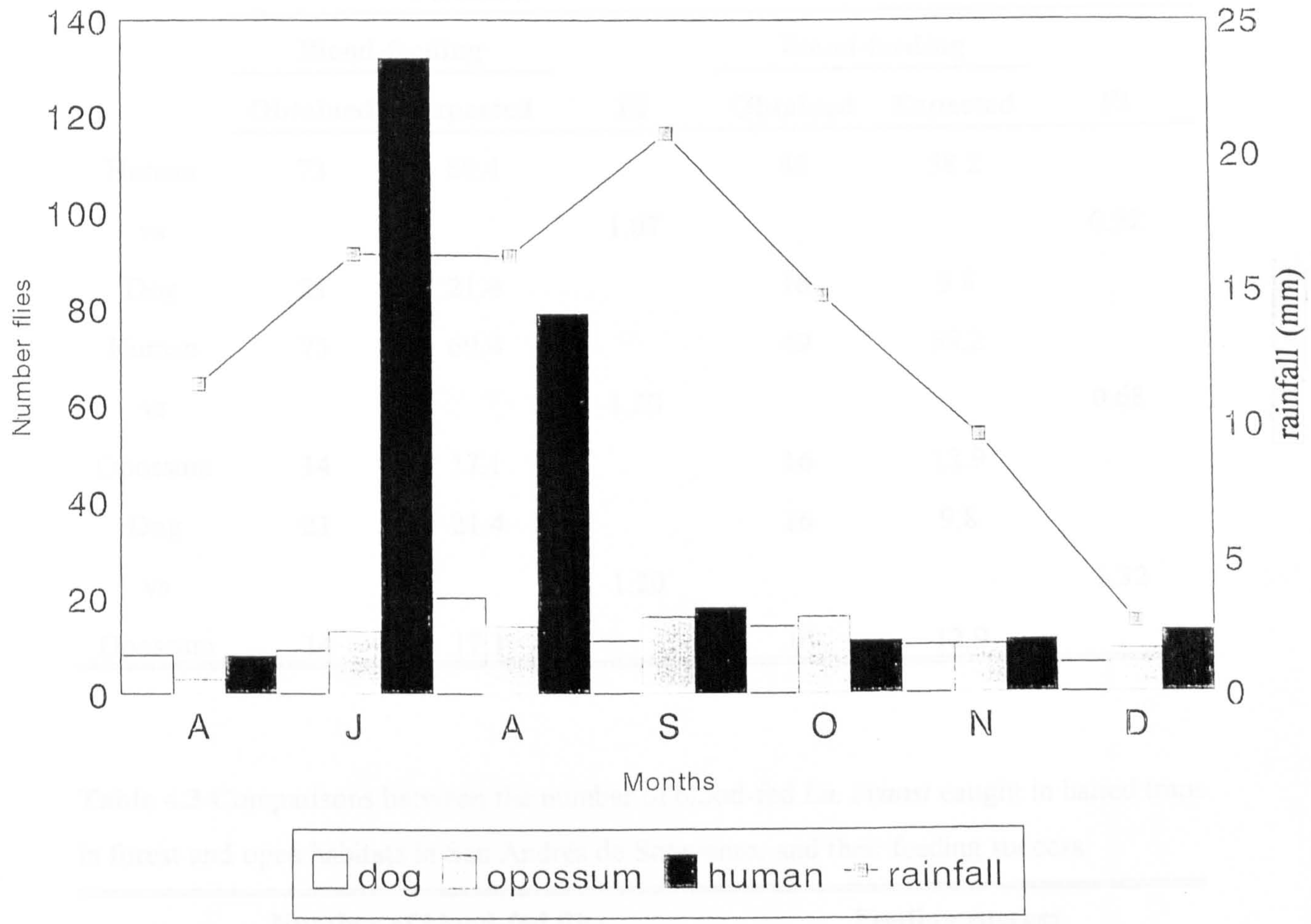



**Figure 4.5**: Number of *Lu. evansi* caught monthly in baited traps between June and December 1992, in El Contento (San Andrés de Sotavento).

When the feeding indices (Table 4.2) were compared, no clear differences between baits were found in the forest site. Feeding indices were similar for all of them. Nevertheless, in the "open" area sandfly feeding preference was greater on dogs and opossums than on humans. Also, a significant difference was found between dog and opossum in this habitat (Table 4.3).

We suspected that host behaviour might be an important factor in feeding success because almost all blood-fed females from human and dog-baited traps (but above all in the former) were found to be fully blood-fed. To test this, all blood-fed females were graded as either partially fed or fully fed. When they did feed on the host, more flies were more fully fed on man and dog than on opossum.

**Table 4.2.** Obtained and expected numbers of *Lu. evansi* feeding on human, dog and opossum and their respective feeding indexes (FI).

| | FOREST | | | NO FOREST | | |
|---|---|---|---|---|---|---|
| | Blood-feeding | | | Blood-feeding | | |
| | Obtained | Expected | FI | Obtained | Expected | FI |
| Human | 73 | 69.4 | | 49 | 58.2 | |
| vs | | | 1.07 | | | 0.52 |
| Dog | 21 | 21.4 | | 16 | 9.8 | |
| Human | 73 | 69.4 | | 49 | 58.2 | |
| vs | | | 1.30 | | | 0.68 |
| Opossum | 14 | 17.1 | | 16 | 12.9 | |
| Dog | 21 | 21.4 | | 16 | 9.8 | |
| vs | | | 1.20 | | | 1.32 |
| Opossum | 14 | 17.1 | | 16 | 12.9 | |

**Table 4.3** Comparisons between the number of blood-fed *Lu. evansi* caught in baited traps in forest and open habitats in San Andrés de Sotavento, and their feeding success.

| | Number of blood-fed flies | | | | Feeding success | | | |
|---|---|---|---|---|---|---|---|---|
| | Forest | | Open | | Forest | | Open | |
| | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p |
| H-D | 0.71 | 0.40 | 8.20 | 0.00 | 1.68 | 0.20 | 8.59 | 0.00 |
| H-O | 5.41 | 0.01 | 2.50 | 0.12 | 17.4 | 0.00 | 0.23 | 0.63 |
| D-O | 1.36 | 0.26 | 1.48 | 0.24 | 5.17 | 0.22 | 4.40 | 0.03 |

4.3.2 Experiment 2: Effect of host and location

In the experiment where domestic animals were placed along transects, individuals of *Lu. evansi*, *Lu. gomezi*, *Lu. panamensis* and *Lu. cayennensis* were found feeding on the animal baits; however the first species was sufficiently abundant for meaningful comparisons. Overall, there was significant difference in the density of female sandflies at different distances from the houses (F= 5.40; df= 2,20; p= 0.1)(Figure 4.6), the low density of flies in sampling

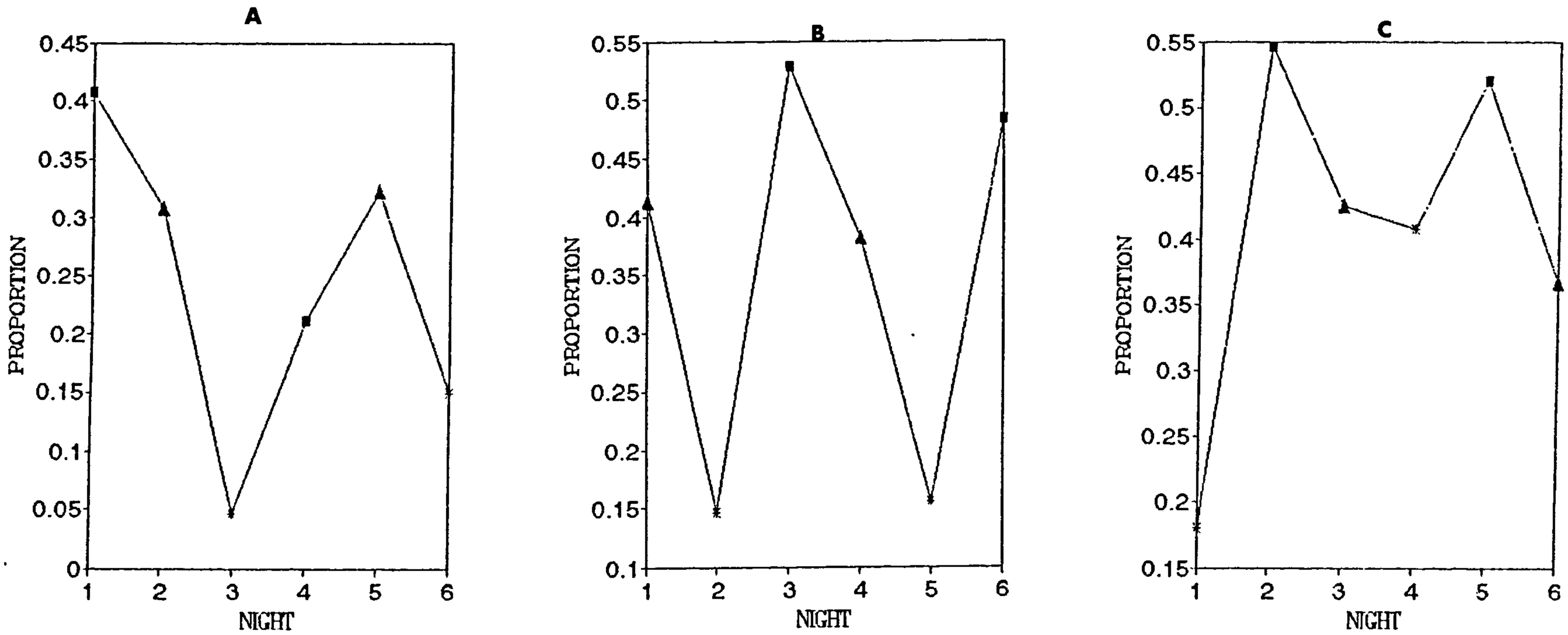



**Figure 4.6** Proportion of flies caught on cows (square); pigs (triangle) and donkeys (asteric) at different distances (A= near (5m), B= middle (20m) and C= away (35m) from houses, for six consecutive nights.

stations near houses being the major source for this variation. Thus, flies were more abundant on hosts in the middle and peripheral areas than near the houses. No additional differences were evident when comparisons between bait were made ($F= 1.37$; $p= 0.05$; $df= 2,20$), though overall a higher mean value of fed flies was picked up on pigs (22.0 flies/bait/night) compared with those recorded on the other two baits (cow= 15.1 flies/bait/night; donkey= 7.4 flies/bait/night) (Table 4.4).

**Table 4.4** Percentage of nulliparous (N), parous (P) and blood-fed *Lu. evansi*, caught on different animal baits n= total flies in samples

| | n | Baits | | |
|---|---|---|---|---|
| | | cow | pig | donkey |
| Nulliparous | 1470 | 36% | 43% | 8% |
| x̄ (± SD) | | 29.9 (26.5) | 35.9 (21.9) | 17.8 (17.6) |
| Parous | 638 | 35% | 44% | 19% |
| x̄ (± SD) | | 12.7 (10.6) | 15.7 (14.7) | 7.9 (5.9) |
| Blood-fed | 786 | 34% | 50% | 15% |
| x̄ (± SD) | | 15.1 (17.8) | 22.0 (22.4) | 7.4 (4.8) |
| TOTAL | 2894 | 36% | 46% | 18% |
| x̄ (± SD) | | 57.7 (48.1) | 73.6 (52.5) | 33.1 (24.9) |
| Ratio N:P | 2.30 | 2.36 | 2.28 | 2.24 |

No statistical differences were observed in the proportion of nulliparous/parous flies between baits. However, the biting parous index (ie. number of parous flies + proportion of blood fed flies) did show a significant trend with more flies further away the houses (away>middle> near). It is unclear, however, whether these are effects inherent to the nature of the baits or related to their position.

4.3.3 Experiment 3: Movement in relation to blood-feeding

In this experiment, on average *Lu. evansi* was the predominant species in all environments (61.8%) followed by *Lu. cayennensis* (33.4%) and *Lu. trinidadensis*. In all species, the highest number of flies (geometric means) were recorded in quadrants nearest to

the houses and abundance decreased as a function of the distance from houses. Unfed *Lu. evansi* (assumed host-seeking) were detected inside and around houses. Fed and gravid females displayed a clear gradient in resting place selection, which varied with the sampling period. ANOVA identified month, distance and collecting method as the main factors affecting male, female and blood-fed sandfly distributions (Table 4.5). Further, goodness-of-fit analysis indicates that flies were patchily distributed well (ie. $\mu < \sigma^2$) with frequencies fitting well the negative binomial distribution ($p > 0.05$) (Southwood, 1980). With very few exceptions, both sticky trap and direct aspiration captures were male biased (Table 4.6). The numbers of *Lu. evansi* females and the proportion of blood-fed increased 2.8 and 4.3 fold, respectively over a 38 day period from the beginning of sandfly activity. Thereafter, they remained at almost the same level (Table 4.7).

**Table 4.5** Factors affecting total numbers of three sandfly species caught by two different methods, between March-May 1994 in San Andrés de Sotavento (Colombia).

| | | *Lu. evansi* | | *Lu. cayennensis* | | *Lu. trinidadensis* | |
|---|---|---|---|---|---|---|---|
| **Factors** | df | F | p | F | p | F | p |
| Month | 3 | 22.5 | <0.001 | 3.96 | 0.008 | 1.48 | 0.21 |
| Day | 6 | 1.72 | 0.102 | 0.57 | 0.782 | 1.27 | 0.26 |
| Distance from houses | 5 | 7.12 | <0.001 | 1.55 | 0.172 | 1.18 | 0.31 |
| Method | 1 | 40.3 | 0.000 | 13.2 | <0.001 | 10.9 | <0.001 |
| Mon*Dis | 15 | 1.82 | 0.028 | 1.20 | 0.260 | 1.72 | 0.04 |

A total of 435 blood-fed flies (94% of which were *Lu. evansi*) were collected from resting places and sticky traps inside and around the houses. Initially, samples were tested using ELISA kits to detect the blood of the most important hosts of visceral leishmaniasis in the area (human and canines). With the exception of intradomiciliary settings, where human represented the main blood source (11.73%), flies showed low interest in dogs (7.0% of the sample). Mixed meals (human/canine) were detected in very few samples (0.83%) while the rest of the feeds did not react. Subsequently, samples were tested against bovines (one of the most obvious animals in the area), and 76.3% of the total were positive. The combination canine/bovine rendered a higher proportion of positives (3.0%) compared with human/bovine (1.1%). The reverse picture (Figure 4.7) was obtained analysing blood-meals obtained from the longitudinal sampling using intradomiciliary sticky traps (see chapter 3).

**Table 4.6** Relative spatial distribution of male and female sandflies collected by direct search (ds) and sticky traps (st) at different distances from houses in San Andrés de Sotavento (Colombia).

| Species | | sex | AREA | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Inside | 1 | 2 | 3 | 4 | Forest |
| *L. evansi* | ds | ♂♂ | 0 | 209 | 138 | 156 | 216 | N.D. |
| | | ♀♀ | 0 | 105 | 47 | 60 | 67 | N.D. |
| | st | ♂♂ | 64 | 261 | 114 | 77 | 53 | 127 |
| | | ♀♀ | 45 | 48 | 17 | 14 | 10 | 20 |
| *L.cayennenensis* | ds | ♂♂ | 0 | 162 | 126 | 116 | 37 | N.D. |
| | | ♀♀ | 0 | 85 | 52 | 52 | 104 | N.D. |
| | st | ♂♂ | 25 | 60 | 55 | 27 | 24 | 101 |
| | | ♀♀ | 46 | 23 | 31 | 27 | 19 | 86 |
| *L.trinidadensis* | ds | ♂♂ | 0 | 13 | 19 | 7 | 19 | N.D. |
| | | ♀♀ | 0 | 11 | 7 | 3 | 15 | N.D. |
| | st | ♂♂ | 2 | 4 | 9 | 1 | 0 | 12 |
| | | ♀♀ | 8 | 2 | 4 | 1 | 1 | 5 |

Areas 1= Quadrats A1-B1-C1-D1. 2= A2-B2-C2-D2. 3= A3-B3=C3-D3. 4= A4-B4-C4-D4. ND= Not done

**Table 4.7** Variation in sex ratio(females/total captures) and percentage of blood-fed flies the main two sandfly species caught over two months in San Andrés de Sotavento (Colombia).

| | *Lu. evansi* | | *Lu. cayennensis* | |
|---|---|---|---|---|
| DAYS | ♀♀: ♂♂ | % BF | ♀♀: ♂♂ | % BF |
| 1-8 | 0.09 | 0.007 | 0.45 | 0.01 |
| 30-38 | 0.25 | 0.03 | 0.26 | 0.16 |
| 40-48 | 0.24 | 0.24 | 0.35 | 0.04 |
| 49-60 | 0.24 | 0.22 | 0.28 | 0.18 |

Surprisingly, a total of 49.2 % of the blood-meals sampled did not react to either antibody tested, making it impossible establish their blood source. Three main factors might account for this failure. Firstly, bacterial deterioration of the sample; secondly it is likely that the non-reactive samples correspond to insects with small, old or partially digested blood-meals. Finally and most likely since all meals in flies were bright-red, samples might also represent baits (such as porcine, avian, rodentia, etc.) which were not included in our screening.

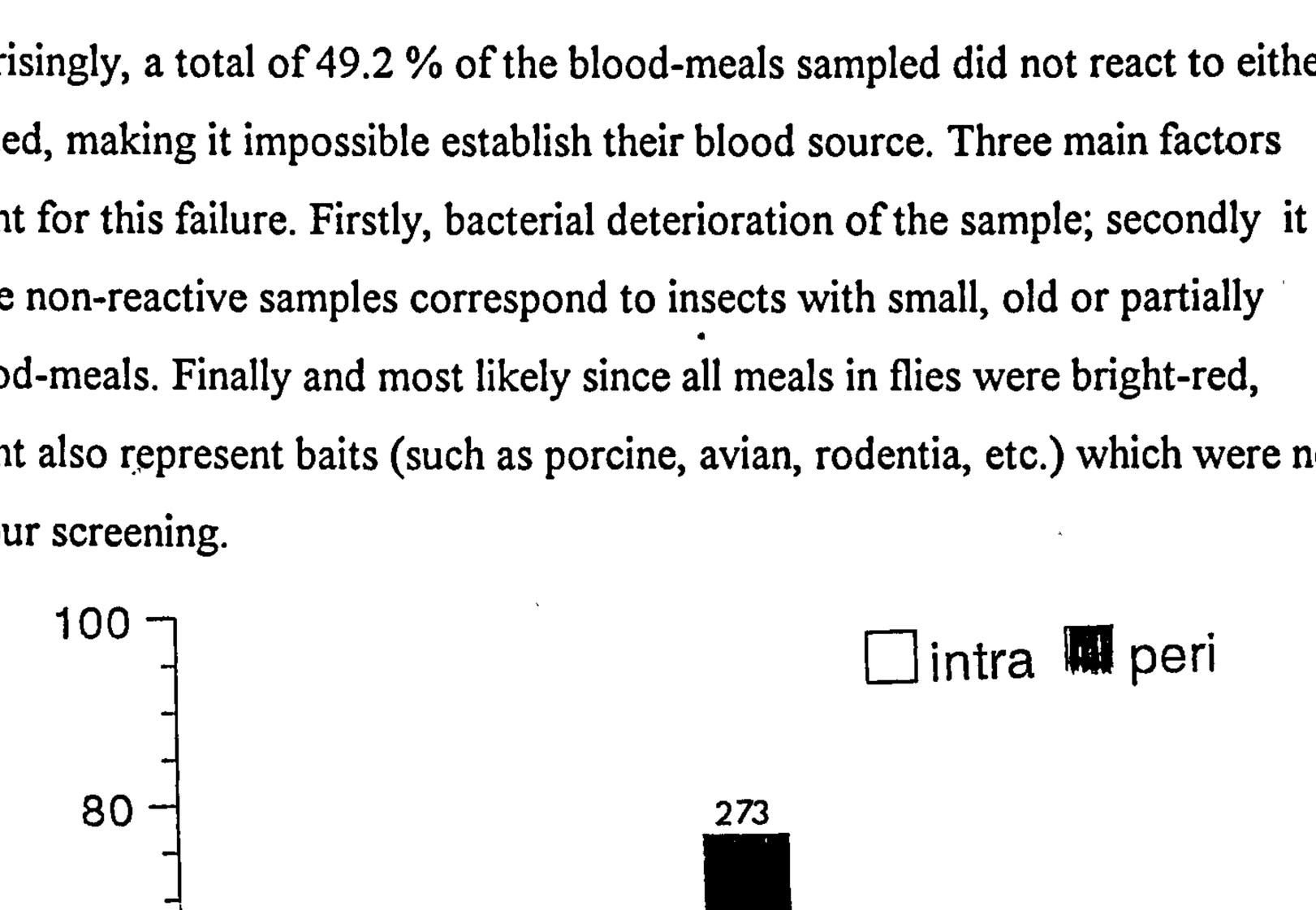



**Figure 4.7**: Blood-meal identifications from blood-fed flies caught inside houses (intra) and around houses (peri).

As it was indicated earlier (Chapter 3), sandflies in El Contento showed preference for some tree species as resting places. In the context of the active searches for sandflies in this experiment, the common tree *G. ulmifolia* and *C. pentandra* had particular importance. These two species were the most preferred resting places for blood-fed and gravid flies. Nearly 80% of the fed and gravid *Lu. evansi* female were picked up from them.

### 4.3.4 Experiment 4: Human-vector contact

A total of 1261 sandflies of five species were caught landing on people. Namely, *Lu. evansi*, *Lu. cayennensis*, *Lu. gomezi*, *Lu. panamensis*, and *Lu. trinidadensis*. Overall, *Lu. evansi* represented 89.1% of these captures. Insufficient numbers of *Lu. trinidadensis* and the two anthropophilic species *Lu. panamensis* and *Lu. gomezi* were caught to enable analysis of their nightly distribution. Hence, the night patterns of the two most abundant intradomiciliary host-seeking flies only were compiled.

*Lu. evansi*: Females represented 88.5% of the total (n=1124) caught alighting on humans. After plotting a histogram of the monthly number of flies/hour/man, it became evident that results followed a skewed distribution (Figure 4.8), October being the month with highest sandfly activity inside houses. In other words, the landing rates showed a seasonal variation. Furthermore, they also varied nightly according to the season. For instance, in April and November a unimodal peak of activity was recorded between 20:00 and 21:00h, with landing rates ranging from 0.89 to 1.15 flies/hour/human. After the peak, sandfly activity was very low, almost nil for the rest of the night in April while low but still some activity was recorded in November. Interestingly, this pattern did not occur during the months with the highest sandfly landing rates (August and October). In August, the main peak begun one hour later than that observed in April and November and lasted for three hours (21:00 to 23:00h) with a secondary peak in the early hours of the morning (04:00). October was the month with the highest and most intense sandfly activity. Biting peaked at 20:00 and continued till 23:00h but two secondary peaks were also seen; the first at midnight and the second early in the morning (04:00h). To generalise, biting activity began at sundown or shortly thereafter, reaching a maximum between 20:00-21:00h. With the exception of April, flies were active throughout the entire night with nearly 80% of the

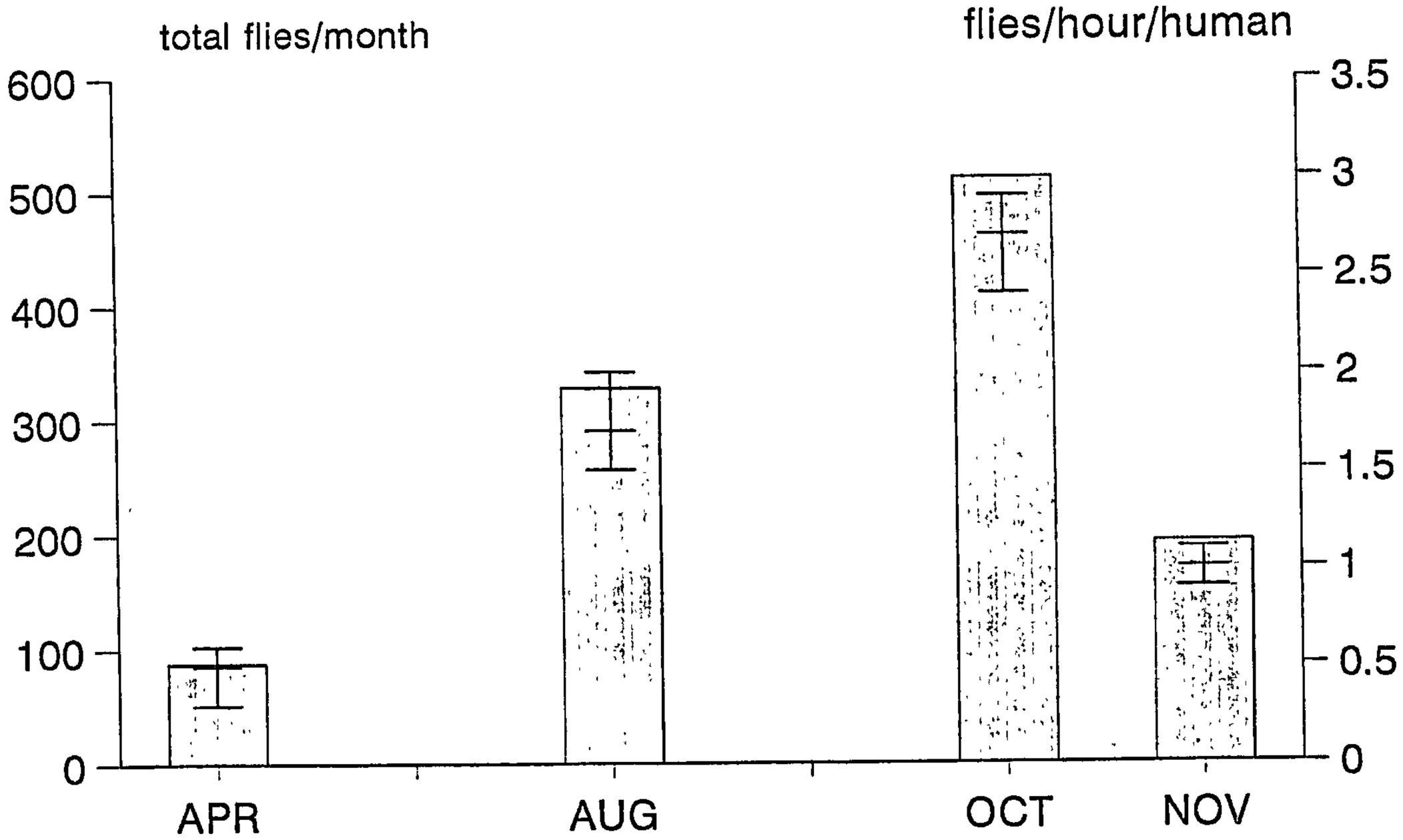



**Figure 4.8**: Histogram of number of flies caught on human bait for four months in El Contento. Bars indicate 95% confidence intervals of biting rate.

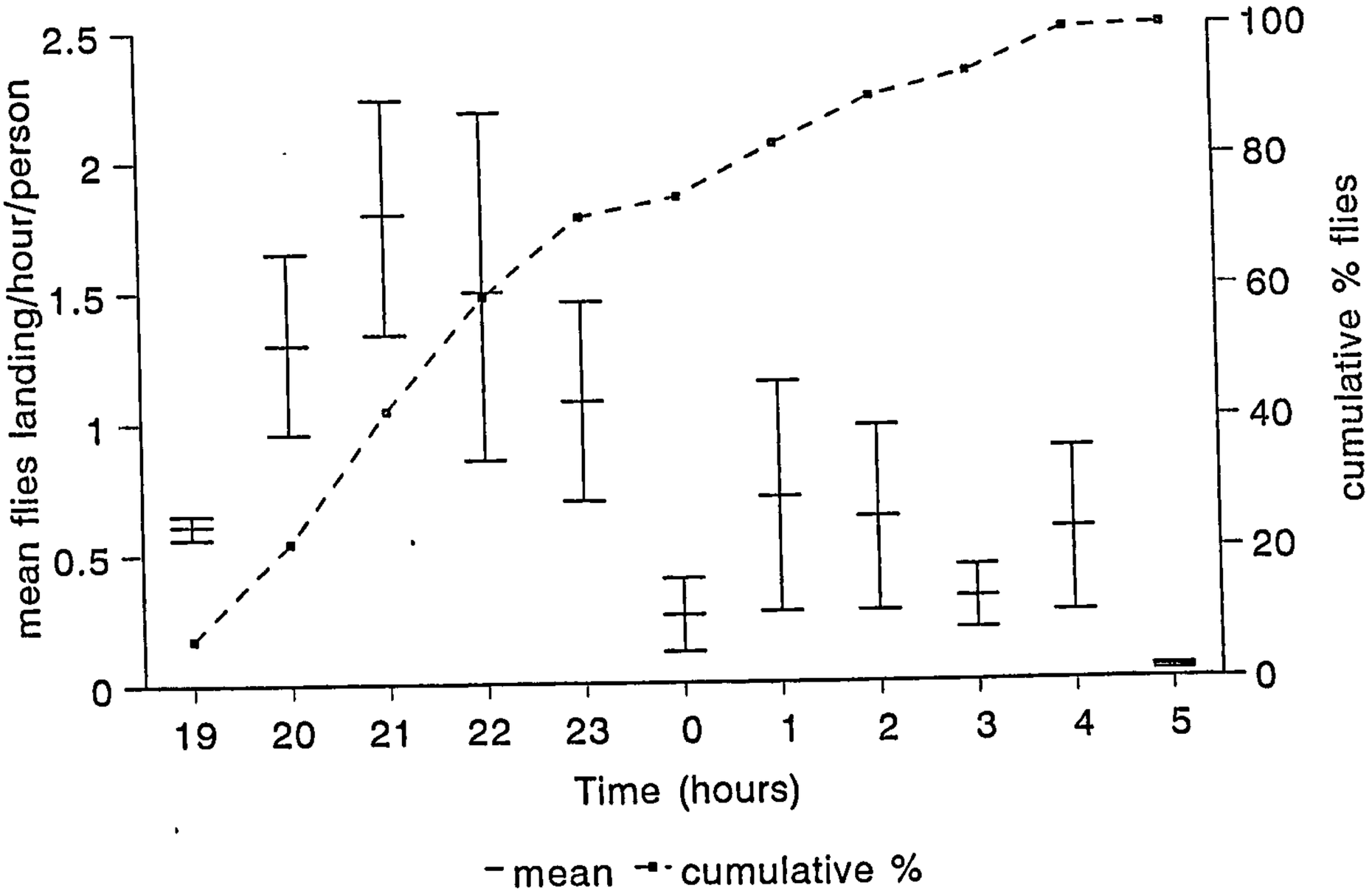



**Figure 4.9:** General human biting activity pattern exhibited by *Lu. evansi* in the San Andrés de Sotavento area.

total of sandfly activity occurring before the midnight (Figure 4.9). Sandfly activity stopped immediately before sunrise (ca. 05:30h).

Contrary to our expectations no resting flies were captured on the inside walls of houses on 'furniture'. This was an unexpected result since dissections of flies revealed the presence of some gravid females inside the human dwellings. Overall, values of monthly parity rates were comparable, with nulliparous flies always outnumbering parous flies. It is noteworthy however, that during October some differences arose in parous rate distributions night to night. More parous females were found in samples captured in the two latest quarters of the night than in samples taken during the two first. However, these differences were not statistically significant.

*Lu. cayennensis*: A total of 82 flies of this species was caught on human, but 40% of them were males. The nightly activity of this sandfly was restricted to the first quarter of the night with an early and unimodal peak (19:00- 20:00). Interestingly, this species, widely thought of as a lizard feeder was found attempting to bite humans. While no discernable differences were found between forest and non-forest sites in the landing activity of *Lu. evansi*, *Lu. cayennensis* was more active in forest settings.

## 4.4 Discussion

From the results, it is amazing to note how "plastic" but at the same time complex, is host-preference behaviour in sandflies. A multiplicity of environmental variables has been reported as affecting the host selection and feeding behaviour of sandflies including the size, abundance and behaviour of the host; the habitat and abiotic factors such as temperature and wind. In the present study, basic assumptions and efforts to control and reduce the main potentially confounding factors were done throughout the experiments. Factors such as trapping site, day to day variation and residual odour between hosts were eliminated by daily rotation of the baits and, when necessary, together with their traps (including plastic ground-sheet as with the cone-traps). Also, potential biases due to abundance of the hosts were controlled as much as possible by holding domestic animals remote from the sampling sites. The importance of atmospheric effects (Varley et al.,

1973) on the observed variations cannot be disregarded. However, these effects are expected to affect all treatments of the experiment in the same way. The discussion will stress, therefore, biological or ecological sources of heterogeneity.

Our results confirm that the dominant sandfly species in the visceral leishmaniasis focus of San Andrés de Sotavento is *Lu. evansi*, with other species poorly represented in almost all domestic environments. Early studies (Travi et al., in press) in the same area demonstrated a similar pattern with *Lu. evansi* constituting 92% and 97.3% of the total catch on human bait (n=909) and in Shannon traps (n=6,697), respectively.

The bimodal seasonal distribution in sandfly abundance measured by the newly developed cone-trap generally follows that reported by Travi et al. (in press) and results of Chapter 3 using a number of techniques. This indicates that the cone-trap does not introduce any systematic bias into the sampling. The highest number of flies were caught at the beginning of the rainy season followed by variable, but sustained, activity of the flies during the rainy season before the numbers fell away in the dry season. Attempts to conduct experiments during the summer period (February-March) were abandoned because the flies were so scarce.

Female flies trapped on human or animal baits as well as those from cone-traps were assumed to be actually host-seeking. In each case there are pieces of evidence that gave support to this assumption. Firstly, the great differences between the control and other cone traps. Secondly, female flies, which predominate in human and animal captures, fed on the baits if the opportunity was given. The presence of blood-fed females in baited traps or direct aspiration catches on baits, confirms the female's attraction to a specific host.

Quite clearly the cone-trap sampling showed that overall there is a preference for humans over either dogs or opossums ($p<0.05$) (reservoirs of *L. chagasi*), and that there is no measurable difference between dogs and opossums (Table 4.8); curiously, these preferences are not equally distinct over all sandfly densities (Figure 4.5). Thus, at low levels of sandfly abundance there is no statistically significant preference for a host but when the sandfly abundance is high (July and August), there is a marked preference for humans. These results indicate that at low densities the sandflies are opportunistic feeders.

It has been postulated that attraction to humans by some visceral leishmaniasis vectors (eg. *Lu. longipalpis*) is a function of the size and number of hosts in the area rather than an intrinsic attraction to humans (cf. Quinnell et al., 1992; Morrison et al., 1993b). Although host preferences may well be affected by abundance of hosts, our results suggest that in the case of *Lu. evansi* preference is independent of size. If host size was the most important factor then, human and cow should always be the preferred hosts in experiments 1 and 2, respectively. However, as indicated by the results (Figures 4.5 and 4.6, respectively) that was not the case in either event. Preference independent of host size in sandflies has been suggested by Christensen & Herrer (1980), Shaw & Lainson (1968) and Mutinga et al. (1986). It is likely that additional factors (intrinsic and extrinsic) have more weight in the modulation of host preference in sandflies than the body size of the host. Recently, in a well controlled experiment, Quinnell & Dye (1994) found that the relative accessibility of the hosts more crucial than its size for *Lu. longipalpis*. In our experiments, pigs attracted many more flies than the bigger baits (cows and donkeys). Hairlessness might be an important in factor in the choice of the host, and certainly in feeding success.

Host and habitat were the main factors associated with variations in attractiveness. In cone-traps, there was a distinct hierarchy in the number of blood-fed flies found in the traps: more flies fed fully on humans than on dogs and, finally, opossums. In the second experiment, the number of fed flies was significantly higher on pig than cow and donkey. Finally, ELISA analysis of flies from sticky traps and direct aspiration revealed bovines as the main blood source for sandflies (anti human, canine and bovine tested). However, since many flies which were collected on pigs were partially fed (second experiment) we suspect that blood from pigs might represent considerable proportion of either mixed or unidentified (or both) blood-meals. It is remarkable how generally similar are the results of the host-preference of *Lu. evansi* in our study and that of *Lu. longipalpis* by Morrison et al. (1993b). According to these authors, *Lu. longipalpis* prefers cow/pig as the main blood sources in the El Callejón focus while donkey, dog, opossum and human were relatively unattractive to the flies. However, in our study area there are fewer domestic animals compared with the Callejon area. That might explain the low intradomiciliar activity recorded for *Lu. longipalpis* in that area, in contrast with the high intradomiciliar pattern of *Lu. evansi* in SAS. These results highlight the difficulty of categorising biting flies as highly or rarely anthropophilic since this behaviour is not just the propensity of flies

to feed on a host (as determine by host choice experiment) but must also take into account the abundance and accessibility of hosts (reflected in the analysis of blood meals from resting flies). Hence, in spite of our results *Lu. evansi* cannot be placed in the rank of 'very anthropophilic' species (like some *Phlebotomus* and *Anopheles* species), although it is clear that its eventual contact with humans appears to be significantly higher than that reported for *Lu. longipalpis*.

All these results strongly suggest that *Lu. evansi* has considerable inter-bait traffic, which might have significant epidemiological implications. According to Bray (1982), an ideal reservoir for *Leishmania* should not only be attractive to a sandfly vector (measured in our case by the relative number of flies caught in the traps of each bait) but, also be the major source of blood-meals. However, in San Andrés de Sotavento although opossums and dogs displayed the highest rates of *L. chagasi* infection (Travi et al., 1994) our results suggest they did not represent the main source of blood for *Lu. evansi* (total number of blood-fed flies on opossums is less than the two other hosts tested). This apparent paradox can also be deduced from the data of Morrison et al. (1993b) working in the VL focus in Melgar, Colombia and was noted in Brazil by Quinnell et al. (1992) who found low attraction to dogs but a high level of infection.

Part of the explanation for these observations can be gleaned from closer examination of the amount of blood consumed by the fed flies in the traps. When flies did manage to feed on a host, more flies were fully fed on passive hosts (humans and dogs) than on opossums (Table 4.3). We interpret these results in terms of the defensive ability of the hosts. Opossums are more nocturnally active than humans or dogs and therefore during the night time, when the experiments were conducted, were in a better position to defend themselves against sandfly feeding. Wild caught *Lu. evansi* females fed more avidly on restrained or anaesthetized opossums than they did on wild rodents, dogs and unrestrained opossums (Travi & Montoya, unpublished observations). A similar phenomenon was found in mosquitoes by Day & Edman (1984).

Therefore, the relative rarity of human cases in the presence of relatively high level of sero-positive dogs cannot simply explained in terms of eclectic feeding behaviour of *Lu. evansi* on animals not susceptible to *L. chagasi* infection (eg. pig, cow, donkey). Thus, in the event of an infected sandfly reaching the human environment it is likely that its infective potential might be diverted to hosts other than either humans or dogs. The same analysis it is more dramatic if applied to sandflies other than *Lu. evansi*. Hence, the role of

other sandfly species as vectors of AVL in SAS can be considered as almost negligible with little, if any, contribution to disease transmission. Relative host attractiveness deserves further study, in connection with the potential zooprophylactic control of visceral leishmaniasis in SAS. Little is presently known about the potential role of domestic animals as diverting targets in leishmaniasis. However, this strategy successfully controlled virus transmission in Kowanyama, Australia (Kay et al., 1979) where the presence of dogs distributed among inhabitants, effectively reduced the arbovirus transmission potential of the vector *Culex annulirostris*.

We assume that gender biases in sandflies observed in our experiments are a direct reflection of their different behaviour. As noted earlier, the predominance of females on sticky traps and in human and animal catches (Experiment 1) can be regarded as evidence of sandfly host-seeking behaviour. In contrast, the high proportion of males in direct aspiration catches is an indication of either mating or resting behaviours. When the sex ratio was dominated by females (eg. Exp. 1), it is plausible to infer that *Lu. evansi* does not form large aggregations of males on hosts as seen in *Lu. longipalpis* and *P. argentipes* (Lane et al., 1990), assuming that the trap design did not interfere with male location of hosts. Further, it is likely that the lack of males in the baited traps indicates that females are not simply responding to male aggregations. However, in this context it is important to note that Galati (cited by Ward et al., 1993) found sex pheromone disseminating structures in *Lu. evansi* (probably from Venezuela) indicating that males produce semio-chemical signals to attract their females.

There are other important issues derived from our results on the study of resting places and host selection which deserve further discussion. Firstly , the contrast between day-time resting and nightly activity patterns in sandflies. Although it was observed that flies do not rest during the day in open areas, at night they are abundant there. In the cone-trap captures, this phenomenon was most apparent during the months of July, August, September and December. One possible explanation for this is the comparative ease of detecting host odours in open compared to forest areas. Although we did not measure odours in our experiments, their role in host detection by mosquitoes has been reviewed by several authors (Hocking, 1971 and references therein).

Another important issue stems from the apparent incongruence in the results of blood-meal analysis and the distribution of resting sandflies. Direct searching collections indicate that most engorged flies rest near houses in the forest areas. However, according to night bait captures, flies were markedly more active in open areas than in the forest. If the flies only travel a very short distance from the host to resting sites then the majority of resting flies it would be expected in trees in the open area. However this was not always the case. Thus, it appears that fed flies fly from open to sheltered places, travelling on average 250m. This might represent a difficult task since a blood-meal represents a heavy load for sandfly flight. This is even more striking considering that *Lutzomyia* species are deemed to be weak fliers (Alexander, 1987; Alexander & Young, 1992; Chianiotis et al., 1974). Although we have no information about the maximum distance flown by engorged *Lutzomyia*, we expected it to be short. So, how do blood-fed *Lu. evansi* face this problem? One possibility is that flies reduce their loads by diuresis, a mechanism widely used by mosquitoes (Clements, 1992; pp: 223; 307-308) allowing excretion of half the weight of the blood-meal within two hours of feeding. A second but less likely explanation, would be that flies take small blood meals. This event is feasible if one considers that dissected indoor caught females were very often partially fed and, sometimes, gravid or semi-gravid. If this occurs, a physiological mechanism might exists controlling host-seeking behaviour in *Lu. evansi* as the hormonal "switch" reported in mosquitoes by Klowden & Lea (1978). According to these authors, the hungry response is only turned off if the insect gut has been expanded enough to reach a threshold controlling the whole system. It is claimed that this mechanism might be the cause of multiple feeding and multiple probing behaviours of some haematophagous species. Since the epidemiological implications of multiple probing or feeding are obvious it would be of interest to pursue studies on this particular subject in sandflies especially, to explain the rare event of gravid *Lu. evansi* trying to bite humans inside houses.

In summary, from these studies, which covered rainy, dry and transition periods in the San Andrés de Sotavento focus, it is concluded that: 1) the majority of sandfly species have a patchily distribution, with more flies around human dwellings; 2) this distribution is determined to a great extent, by the presence of potential blood sources in the area (pigs, cows, donkeys, dogs, humans, etc.) and the availability of suitable resting places, represented by some tree species; 3) differences between catches on hosts in the forest and

open habitats (e.g. blood feeding), though these were not consistent or systematic in nature; 4) there were differences in host preferences and, 5) blood meal identification analysis shows that despite the high endophilic activity of *Lu. evansi* despite its avid biting of humans it is not a "highly anthropophilic" species (cf. Feliciangeli et al., 1992; Zeledón et al., 1984; 1989 and Young and Duncan, 1994; Travi et al., in press) but, should be considered as an eclectic feeder.

# CHAPTER 5

# SANDFLY MOVEMENT

## 5.1 Background

The study of flight movements of insect vectors of diseases is important from a number of viewpoints. Among the most important are: the distance regularly travelled by host-seeking females; the furthest distance flies can travel (dispersal); age dependent dispersal, and movement between isolated populations and its role in gene flow. One of the most direct ways to measure insect movement is through marking-release-recapture experiments (for a review see Southwood, 1980 and Hopper, 1991). Actually, marking techniques provide the simplest, most accurate and inexpensive methods to study insect movement under field conditions. However, the low recovery rate frequently found in practice is their main weakness.

Although extensively used in medical entomology studies, mark-release-recapture techniques have been rather poorly explored in the study of sandfly populations. In addition, it is not always possible to compare the results of the few studies that have been made because the experimental designs vary so much. The main differences are in the distance of trapping from release points, the conditions of the released flies, and arrangement and method of trapping. However from the few studies that have been made it appears that Old World *Phlebotomus* species move further than their New World counterparts, *Lutzomyia*, which are more passive flyers usually remaining close to "breeding/resting" sites. But if the ecological conditions of the places where the studies were carried out are taken into account then it seems that dispersal is greatly affected by the physical structure of the environment. In other words, some species are "obligated" to fly further in search of scarce hosts and resting sites. For instance, it has been reported that in a barren land (in Uzbekistan), *P. papatasi* can fly up to 4km (Streklova & Kruglov (1985) (cited in Alexander & Young, 1992)) but, Doha et al. (1991), working in El Agamy on the Mediterranean coast of Egypt, found that unfed *P. papatasi* and *P. langeroni* females travelled distances of only around 900 and 400m, respectively. Most of the engorged females they released stayed near the release point until

blood-meal digestion was completed. Most males preferred to stay near the release point, not dispersing > 600m, while a few travelled distances > 1400m. In other environments (including human settlements) the flight range of some *Phlebotomus* has not exceeded 1000m; eg. *P. orientalis* in Sudan, moving 740m. (Quate, 1964) and *P. ariasi* in the Cévennes mountains, France, moving 750m (Rioux et al. 1979). In the last area, further well controlled mark-release experiments showed that fed females of *P. ariasi* remained in the release zone in contrast with unfed ones which dispersed more than 2km (Killick-Kendrick et al., 1984).

The above mentioned works provide valuable information on dispersal of both unfed and fed females but they cannot be drawn on to predict dispersal behaviour of Neotropical sandflies. The comparison is not between two genera (*Phlebotomus* and *Lutzomyia*) as generally discussed, but should be focused on response to different habitats. Given the wide range of ecotypes in the Neotropics it is difficult to make generalisations. While in some forest areas (eg. Panama) the complex physical structure of the environment and humid conditions are regarded as factors influencing limited *Lutzomyia* dispersal (Chianiotis et al., 1974) but in crop plantations (eg. coffee growing areas) nutrient availability and a wide choice of resting places are suggested as the principal factors (Alexander, 1987). In less forested areas (eg. Marajó island, in Brazil) flies do not disperse very far (at least in *Lu. longipalpis*) although this is not constant but variable (Dye et al.,1991). In contrast, in semi-arid areas (eg. the AVL focus of the Magdalena Valley in Colombia), the dispersal behaviour of *Lu. longipalpis* is more similar to that of Old World species from arid places than to other Neotropical sandflies (Morrison et al., 1993).

Given the ecological similarities between places where *Lu. evansi* and *Lu. longipalpis* occur in Colombia, we expected the movement and spatial distribution patterns of these two species to be similar. As part of the present study on the biology of the sandfly fauna of the AVL focus of San Andrés de Sotavento, sandfly movement was studied at two different levels. Firstly, a direct estimation of the actual flight range in *Lu. evansi* was obtained based on a mark-release-recapture experiment. The overall rationale of this experiment was to mark and release flies in two sites some 2km apart and then attempt the recapture in both areas detecting local and larger movement (ie. whether flies from vegetation patches move to houses and vice-versa). In the second experiment, small scale movements of blood-fed and presumed "host-seeking" flies were inferred from the distribution of flies in peridomiciliary areas using conventional trapping methodologies (sticky trap and direct search in resting places) combined with ELISA analysis (see Chapter 4).

## 5.2 Methods and Results

### 5.2.1 Laboratory trials:

5.2.1.1 Testing marking procedures: Prior to the field work, two different mass-marking techniques were separately evaluated under lab conditions in London for their ability to mark flies for a long time and their effect on survival. In the absence at that time of colonised *Lutzomyia* species, a colony of *P. papatasi* from Israel was used. The first marking technique tested used an internal dye, Rhodamine B (BDH Limited Poole, England) which is a biological fluorescent stain, soluble in water and with high durability under field conditions (Reeves et al., 1948). Four batches of 75 flies each were used; three of them were exposed to different Rhodamine concentrations (ie. 0.01, 0.1 and 1.0%) in 20% of sugar solution and the fourth was used as a control. Flies had access to dye solutions for 12h, period after which almost all flies were observed to be well marked.

In the second technique, three Day-Glo® fluorescent powders were used: Rocket red (A-13N), Saturn yellow (A-17N) and Signal green (A-18N) (Day-Glo Colour Corporation, Cleveland). The selection of these powders was based on their successful application to previous *Lutzomyia* studies (Alexander, 1987). Batches of 60 3-4 day old flies (sex ratio 1:1) were confined to Nalgene® plastered pots covered with a fine mesh. The flies and the dust (approx. 10mg per /pot) were agitated for about 20s, blowing air with a small hand-held pump via a capillary tube inserted through the holes of the mesh.

In all cases, marked flies were kept in Barraud cages, provided with 20% sugar solution and kept inside an incubator (27°C and 85% RH). The flies were then monitored for mortality and mark durability for 10 days. Unmarked flies (controls) were maintained along side marked flies in the same conditions.

In satellite experiments, the effect of the markers (Rhodamine and powder) on the feeding behaviour of flies was tested. Batches of 151 (for Rhodamine) and 171(for powders) flies were allowed to take blood through a chicken membrane five days after marking. The number of fed flies was recorded and compared with control groups. All results were tested using simple $\chi^2$ test.

Although no significant differences in mortality rates were observed between batches of flies marked with Rhodamine ($\chi^2 = 0.001$; $p = 0.97$), there were differences in dye intake and durability. Flies fed well on the 0.01% concentration but after 4-5 days there was no

signal of Rhodamine in their intestine. In contrast, flies exposed to 1.0% were reluctant to feed but remained marked for a longer time. The optimum balance of palatability, survival and durability was the 0.1% dye solution. From a total of 60 flies examined in this group, 84.6 % remained well marked for 6.5 days. The marker lasted for longer but a UV light source was necessary for its detection.

Overall, fluorescent powders did not significantly affect survival of male or female flies ($p > 0.05$) though some slight differences were noted when a further segregated analysis was done. The mortality rate of flies marked with Saturn yellow powder was almost twice than that recorded in the control group. Other marked flies experienced less than 5% mortality, which was evenly distributed throughout the 10-day period. In contrast to the Rhodamine results, no significant differences in dye durability were observed among the three groups. Regardless of the colour used, traces could be detected even 10days after marking using an UV light.

Finally, none of the markers appeared to affect the feeding behaviour of the flies. Marked flies fed well through chicken membranes showing no differences to respect to the control groups ($\chi^2 = 3.26$; df= 2; p= 0.196).

Given the ease of use, long durability of the marking and lack of any effect on survival of flies, green and red powders were selected for use in subsequent field experiments.

### 5.2.2 Field Experiments

#### 5.2.2.1 Experiment 1: Mark-release-recapture

5.2.2.1.1 Collection and release sites: Field work was carried in El Contento (SAS) during a two week period in July/August 1993, the time of the year when sandfly numbers are at their highest. Two sites were selected for both capture and release sites. The first site was in some relatively unaltered vegetation, and the second approx. 2km away in the main human settlement (ie. surrounded by shrub vegetation) (Figure 5.1). Simultaneously, in the two sites, sandflies were caught during a three hour period on two consecutive nights. Three people per Shannon trap collected sandflies between 20:00 and 23:00 h. Catches were gently aspirated off the sheet of the trap, roughly counted and blown into Barraud's cages inside plastic bags supplied with a damp sponge for humidity. Caught flies were marked with fluorescent powders;those from the first site (forest) were marked with green powder while those from

the second site ('inhabited') were marked with red powder. Different marking pots and equipment were used for each colour. Marked flies were kept inside the pots and then released the same night, near the places where they were captured by removing the lids of the pots and leaving them on the ground.

5.2.2.1.2 Evaluation of field mortality and recapture: After 12h, the pots were recovered and the number of dead and 'disabled' flies recorded. Additionally, before fly release, samples of marked and unmarked flies were taken at random from both populations and kept under lab conditions as controls for mortality and to determine species composition.

Two batches of marked flies were released at each site, one night after the other, ie. four batches released in all. Following release of the marked flies, attempts to recover them were made over ten consecutive nights using direct inspection of resting sites and sticky traps. A total of eight stations were strategically located (Figure 5.1). On a daily basis, in each station 20 sticky traps were set up and left overnight. The following morning they were inspected and the surrounding area was searched for resting flies. In addition, animal bait captures (using cow, donkey and pig) were carried out in stations R1, R2 and R4 for six consecutive days, starting on the third day after sandfly release. Human bait collections were made but in the four stations around the village only (ie. the second release site) for five alternate days.

All caught flies were transported to the field laboratory, placed on a black-background and inspected with an ultraviolet lamp for fluorescence of the marked powders.

The total numbers of caught and marked flies varied slightly between the two sites (Table 5.1). The marked flies were presumed to be *Lu. evansi* as this species accounted for 99% of the flies collected for estimating species composition and evaluation of mortality rates in the lab.

Under field lab conditions, the overall mortality rate was three times higher in marked flies than unmarked (control) (Table 5.2). Since the majority of deaths occurred within the 24h period after marking, these data were used to estimate the number of released flies (corrected) surviving in the field to be recovered.

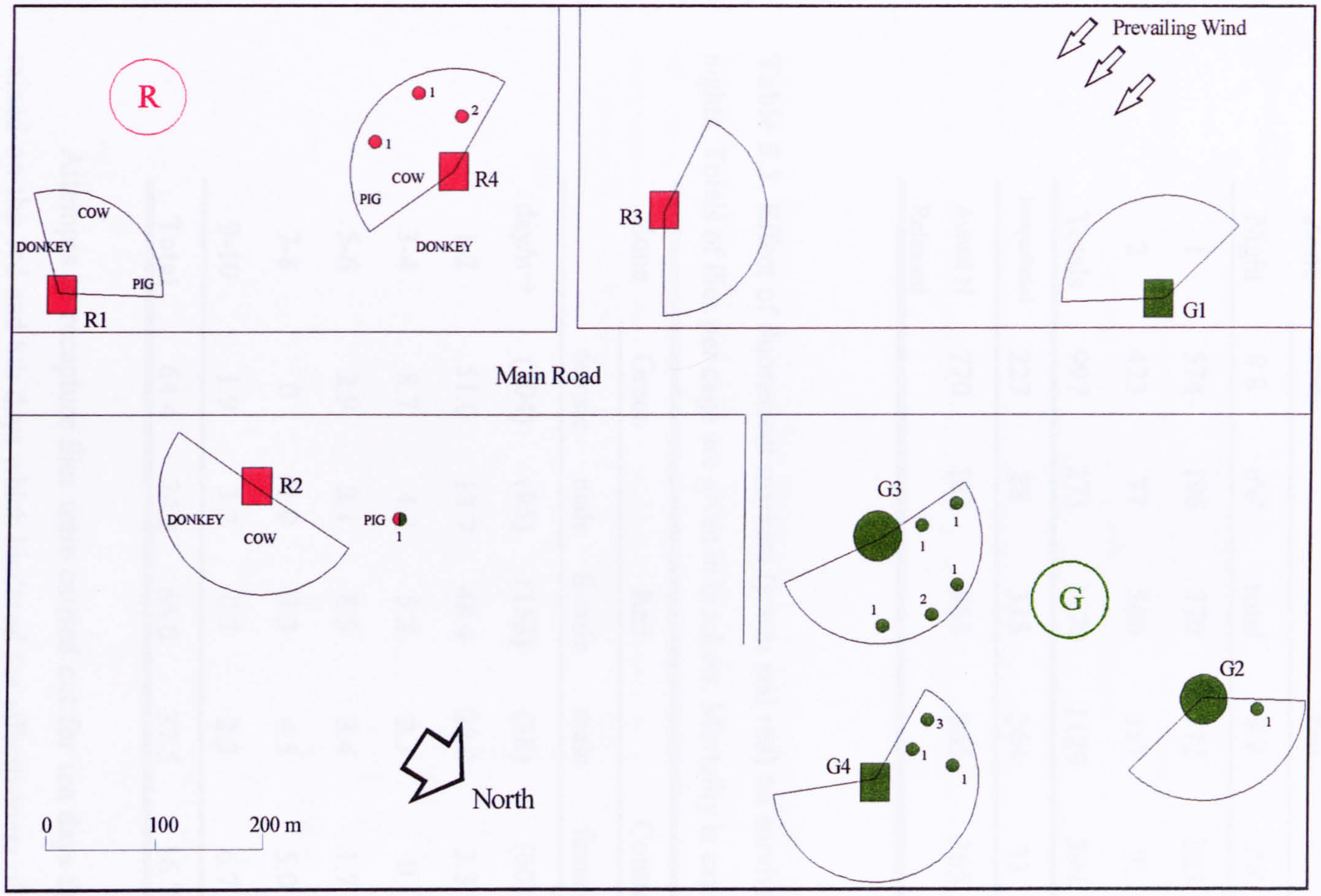



**Figure 5.1.** Diagrammatic map showing the two sites of sandfly collection and release after marking with fluorescent powders (in open circles). Recaptures were made by sticky traps, direct search and animal bait in the segments denoted by $R_1$ $R_4$ and $G_1$ $G_4$. The position where individual flies were recaptured are indicated by small solid circles.

**Table 5.1** Total of female and male sandflies caught in Shannon traps and marked by fluorescent powders (green and red) over two nights of captures.

| Zone | Green | | | Red | | |
|---|---|---|---|---|---|---|
| Night | ♀♀ | ♂♂ | total | ♀♀ | ♂♂ | total |
| 1 | 574 | 196 | 770 | 772 | 323 | 1095 |
| 2 | 423 | 77 | 500 | 357 | 75 | 432 |
| Totals | 997 | 273 | 1270 | 1129 | 398 | 1527 |
| Incapacitated | 227 | 88 | 315 | 264 | 33 | 297 |
| Actual N Released | 770 | 185 | 955 | 865 | 365 | 1230 |

**Table 5.2**: Effect of fluorescent markers (green and red) on survival of sandflies over ten nights. Totals of flies per cage are given in brackets. Mortality is expressed in %.

| Zone | Green | | Red | | Control | |
|---|---|---|---|---|---|---|
| | female | male | female | male | female | male |
| day/n→ | (104) | (95) | (153) | (88) | (60) | (65) |
| 1-2 | 51.0 | 13.7 | 48.4 | 25.0 | 3.3 | 1.5 |
| 3-4 | 8.7 | 4.2 | 5.2 | 2.3 | 0 | 4.6 |
| 5-6 | 2.9 | 2.1 | 8.5 | 3.4 | 1.7 | 6.2 |
| 7-8 | 0 | 1.0 | 3.3 | 4.5 | 5.0 | 1.5 |
| 9-10 | 1.9 | 3.2 | 0.7 | 2.3 | 6.7 | 3.0 |
| **Total** | 64.4 | 24.2 | 66.0 | 37.5 | 16.7 | 16.9 |

Attempts to recapture flies were carried out for ten days following release, but it rained on the 3rd and 8th days which hindered the effectiveness of sticky traps and might have had some effect on the activity of flies too.

With the exception of the two rainy days, winds were virtually absent during the study period and the number of sandflies caught was always in the hundreds per day. The total number of recovered flies was 17, which represented a low recapture rate in both areas (corrected values: green=1.4% and red= 0.44%). Interestingly, direct search for resting flies was overall the most efficient method of recovering marked flies (Table 5.3). This was

particularly true in the forest area, where the majority of recoveries (75%) was obtained by this method. These results contrast with those obtained from the inhabited area, where the number of recovered flies was equal in both sticky traps (2 flies) and aspiration in resting places (2 flies).

As stated above, the most successful method of recapturing flies was by direct search, contrary to our preconceptions. The total number of resting flies examined and the total of recoveries discriminated by day and zone are displayed in Figure 5.2. Overall, more females were caught in the open area than in the forest but there was no such difference in the numbers of males caught. The total number of flies caught in the two areas varied between days. It appears that a decrease in one area was followed by an increase in the other (Figure 5.2).

**Table 5.3**: Totals of marked flies recovered over ten night period. Flies were discriminated by number, sex, method and distance travelled per day. The two areas and markers are identified by G=green and R=red. DS= direct search; ST= sticky traps; AB= animal bait

| Release Point | Recovery Point | No/Sex | Method/Distance | Days since Release |
|---|---|---|---|---|
| G | $G_2$ | 1♀ | DS/ 150m | 2 |
| | $G_3$ | 1♀ | DS/100m | 1 |
| | $G_3$ | 1♀ | DS/110m | 2 |
| | $G_3$ | 1♀ | DS/140m | 2 |
| | $G_3$ | 1♂ | DS/160m | 2 |
| | $G_4$ | 1♀1♂ | DS/150m | 2 |
| | $G_4$ | 1♂ | DS/160m | 2 |
| | $G_4$ | 1♂ | DS/180m | 2 |
| | $G_3$ | 1♂ | ST/75m | 1 |
| | $G_3$ | 1♀ | ST/110m | 2 |
| | $G_4$ | 1♂ | ST/150m | 3 |
| R | $R_4$ | 1♀1♂ | DS/250m | 1 |
| | $R_4$ | 1♂ | ST/190m | 1 |
| | $R_4$ | 1♂ | ST/210m | 2 |
| G | $R_2$ | 1♀ | AB/600m | 5 |

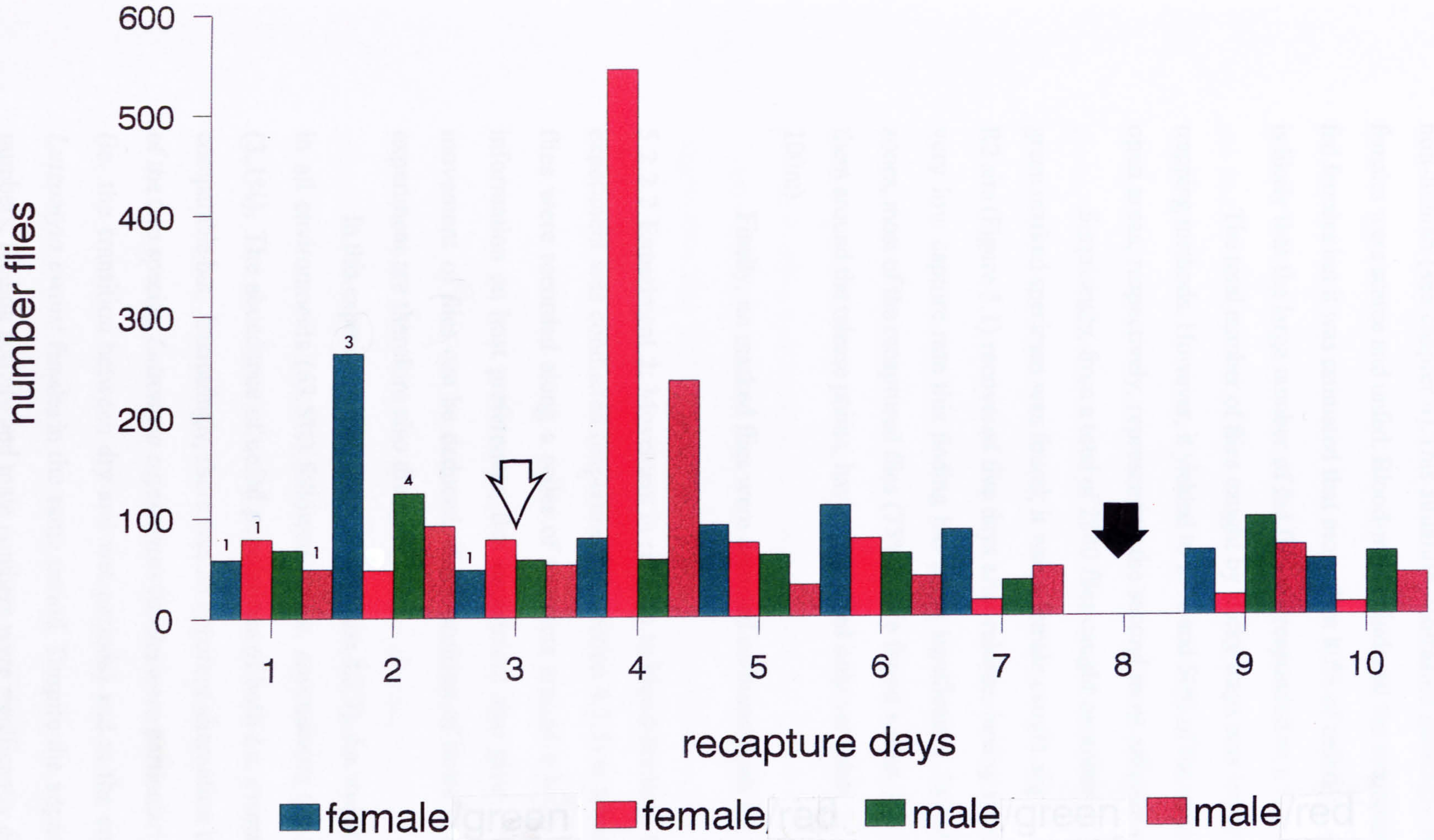



**Figure 5.2** Total of flies collected in resting places through 10d sampling in two areas (inhabited= red and forest= green). Peridomiciliar female flies increased after the cease of a mild rainy night (white arrow), conversely, absolute number of flies in both areas were affected by a second, stormy rainy night (black arrow), numbers at the top of the colums indicate recovered flies.

The sharp increase in flies caught in the inhabited area occurred immediately after the first rainy day (fourth day) and that more than 60% of the collected females (n= 602) at this time were recently blood-fed. ELISA analysis indicated that the origin of the blood source was non-human (see chapter 4).This situation contrasted strikingly with the forest area where females were scarce and unfed. Blood-meals hindered the examination of the parous grade in fed females but it was estimated that more than 80% of unfed flies were nulliparous. Thus, it is likely that the large number of fed flies corresponded to a recent new emergence.

The total number of flies caught by sticky traps was lower compared with the rest of trapping methods. However, it yielded to 25% and 50% of the recovered flies in the forest and open areas, respectively, representing the second most efficient method of recovering flies.

Surprisingly, from a total of 2840 flies caught on animal baits examined, only a single green marked specimen was found; it was a female caught alighting on a pig stationed at the R2 site (Figure 5.1) recovered five days after release, having travelled > 600m . Despite this very low capture rate this finding has some significance. In both the forest and inhabited zones, most of the recaptured flies (75%) were found within 48h of release and almost all of them around the release points, having travelled only very short distances (ie. no farther than 100m).

Finally, no marked flies were detected on human bait captures.

5.2.2.2 Experiment 2: Movement in relation to blood-feeding: In the previous chapter, an experiment was conducted (experiment 3, section 4.2.3) in which the position of blood-fed flies were recorded along a series of transects around a house. In addition to producing information on host preferences, this experiment also gives data from which small scale movement of flies can be deduced, if the position of hosts is known. The results of this experiment are therefore also discussed here.

In this experiment (see Chapter 4, section 4.3.3), *Lu. evansi* was the dominant species in all environments (63.5%) followed by *Lu. cayennensis* (33.4%) and *Lu. trinidadensis* (3.1%). The abundance of unfed populations of both *Lu. evansi* and *Lu. cayennensis* was comparable but, interestingly, there was an apparent alternation between female populations of the two species. *Lutzomyia cayennensis* females were particularly more abundant in March (ie. the transition between dry and wet periods) and at the end of the study (June) than *Lutzomyia evansi* females in the same period. Despite the apparent homogeneity in female numbers, feeding activity and male numbers were significantly different in the two species

throughout the study. More blood-fed females of *Lu. evansi* (Mantel Hanzel $\chi^2 = 33.3$; p <0001) were found in the presence of males of *Lu. cayennensis*. Although males were widely distributed their highest densities were associated with the areas with the highest female feeding rates (Figure 5.3), though this was not obvious at the beginning of sandfly activity (first two months) when few (only 23) blood-fed flies were caught along the zones 2, 3 and 4 at variable distances from the potential blood sources. In the following two months in both species, the highest numbers of flies (geometric means) were recorded in quadrants nearest the houses and abundance decreased as a function of the distance from them (Figure 5.3). Unfed *Lu. evansi*, possibly host-seeking, were detected inside and evenly around houses. Interestingly, freshly fed and gravid females displayed a clear gradient in preference for resting places, which varied with the sampling period but in almost all cases indicated that they were patchily distributed. One group was close to the trees where domestic animals (the most likely blood sources) were kept (zones 1-3). Gravid females were located near the vegetation borders (zone 5) and the forest. The percentage of fed flies during this period was significantly greater (87%) than the previous two months (13%; total n=175 blood-fed flies), partially agreeing with the increases in the number of 'host-seeking' females collected in the same period. Bovines continued to be the main source (43%) but the analysis of the gut contents of flies caught inside houses were mainly of human and canid origin, though meals of mixed origins were also detected, especially inside house and in zones 1 and 2.

## 5.3 Discussion

Despite the fact that results from our trials in London showed that under lab conditions powders did not affect survival of sandflies (at least for *P. papatasi*) it appeared that under field lab conditions, mortality increased in marked and caged *Lu. evansi*. Although generic and physiological differences may have accounted for this disparity in results, stress due to dusting procedure rather than a direct effect of the powders is presumably the main cause of the field mortality rate. After all, no significant differences were observed in mortality rates between control and marked flies during the following 8d period of the field experiment (Table 5.2). In this regards, our results agreed well with those for marked *Lu. longipalpis* recorded by Morrison et al. (1993b), who noted a significantly higher mortality rate on day 1 compared with unmarked colonized flies. For the analysis of the field experiments a conservative estimate of the number of surviving flies was used. In other words, only those

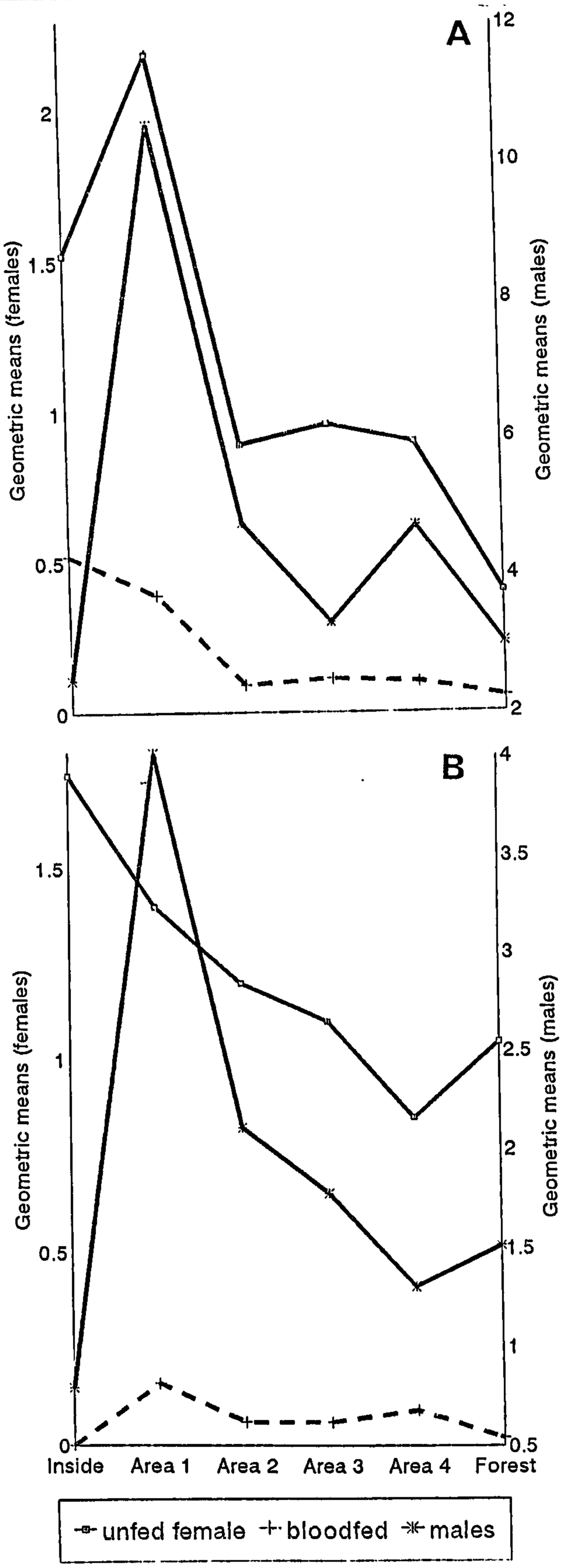



**Figure 5.3** Comparative geometric means of females (unfed and blood-fed) and males of (A) *Lu. evansi* and (B) *Lu. cayennensis*caught resting at different distances from houses (pooled data).

well marked but healthy flies were used for the analysis of recapture rates.

Before further discussion, it is important to note that all field marking-release experiments introduce artifacts, often precluding meaningful comparisons or making analysis difficult. Even assuming that marking did not affect sandfly survival and that powders were not lost from flies, there are still factors that might have biased the results. For instance, although the number of marked flies in our studies did not depart substantially from those used in other marking-release-recapture experiments in Colombia (cf. Morrison et al. 1993; Alexander 1987) it is likely that to some extent the low recovery rate in El Contento area is attributable to dilution of the marked flies due to emergence and migration (Curtis & Rawlings, 1980). These authors estimated a decline of about 25% per day in the percentage of marked anopheline mosquitoes in a population. In our case, the dilution effect may be especially high, considering that experiments were done during a period when the adult fly population was expanding. The recapture of a green marked fly on day 5 in the red area when relatively few marked flies were expected to reappear reinforce this point. Even if the sandfly population was stable and that there was no dilution, it would be very difficult to sample in all possible directions in which sandflies could disperse. It is noteworthy that despite the recapture efforts being focused on the open area, the majority of marked flies were caught in the forest and no further than 150m from the release point (Table 5.3). This was contradictory to expectation. As pointed out Alexander & Young (1992), this kind of difference may reflect the occurrence of irregularities in behaviour of marked flies and that, for instance, if host seeking flies are prevented from feeding they may prefer instead remain at rest until a total physiological recovery takes or when natural conditions become favourable again. Although such effects were not observed in our feeding trials using bred *P. papatasi* we have insufficient evidence that this did not occur in field *Lu. evansi* populations.

Overall, our results on mark-release-recapture were more consistent with that reported for forest sandflies (cf. Chianiotis et al., 1974; Chippaux et al., 1984; Alexander et al. 1987 and Alexander & Young, 1992) and for *Lu. longipalpis* in Brazil (cf. Dye et al., 1991) than those reported for *Lu. longipalpis* by Morrison et al. (1993b). This is particularly amazing since in Colombia, both *Lu. evansi* and *Lu. longipalpis* occur (though separately) in semi-arid forest environments. However, given that in our study only a very low fraction of marked flies were recovered during the first experiment and the obvious limitations of the second experiment direct comparisons are not realistic. Besides, there are serious constraints on the

use of bred flies in field experiments as used by Morrison et al. (1993b).

Only a few cautious conclusions based on the most tangible results can be drawn from our analysis combining both the mark-release and blood meal analysis of the transect experiment. A first and most obvious conclusion is the corroboration of our previous results on the clumped distribution and movement of flies in the area. These two aspects appeared to be affected by the availability of both hosts and resting places in the area. In the mark release recapture experiment, marked flies did not move far, the majority recoveries being at a mean distance of 175m from the release points. Further indications of this effect came from the patchy distribution of blood-fed females around houses in the second experiment. However, in the latter case there were strong indications that flies might have moved, sometimes more than 100m in their search for human and domestic animals. In the first experiment, the main source of the freshly blood-fed flies collected on day four in the inhabited area was identified of bovine origin. In contrast, in the forest environment this was not detected for a further two days later (ie. 6th day). Since blood resources in the forest area are mainly represented by small rodents and marsupials (G. Adler, pers. comm.) flies might have flown some distance in search of alternate hosts. Not surprisingly, blood-meal identification of the stomach contents of flies indicated that the majority of them fed on domestic animals, probably on those around domestic settings (red area). Finally, in May and June, resting site collections indicated that zone 5 and the patch of forest were the preferred areas for gravid females which suggests movement from peridomiciliarysettings (ie. inside and zones 1-3) to these areas.

The available evidence here and that of other studies on *Lutzomyia* species (cf. Alexander, 1987; Dye et al. 1991) suggests the following movement pattern for sandflies in El Contento area. Firstly, with the start of the rains, the sandfly population increases in the forest, males and unfed females are seen in almost all areas. Nulliparous females move mainly towards the inhabited areas, probably in search for potential blood sources, as indicated in the second experiment. Males and freshly engorged females remain around blood-meal sources moving only short distances (no more than 250m) to resting sites. Gravid females return to forest patches looking for suitable resting/breeding places moving between 100m and 1000m. Although sandfly activity is depressed by strong gusts, mild winds may assist this dispersal. Therefore, the recapture of marked fly beyond 800m (first experiment), is perhaps the typical flight range of *Lu. evansi* in El Contento area.

Two immediate implications for the dispersal and commuting ability of *Lu. evansi* between forest and open areas and its endophilic but exophagic (egg maturation) behaviour. Firstly, all of these show clearly that this sandfly species is well adapted to disturbed habitats around human dwellings. Hence, we conclude that any attempt to control this fly by clear felling of forest (cf. Esterre et al., 1986) is unlikely to be successful. Similarly, the exophagic behaviour displayed by *Lu. evansi* would decrease the efficiency of any house-spraying programs. This is even more striking given that in the field there is no clear line demarkation between the habitat around houses in forested sections of the villages and those in cleared areas in the middle of villages. As described earlier (Chapter 2), the forest in this part of Córdoba Department is dry forest, more akin to scrub than the dense, high and wet forest elsewhere in Colombia (eg. Bajo Calima or Tumaco; cf. Travi et al. (1988)) where such a difference between village centre and periphery is more overt. Secondly, eventhough a firm conclusion based on the recapture of a single specimen cannot be made, the finding of this fly six to seven days after its release indicates the survival period of wild *Lu. evansi.* According to this, females can survive at least for 8 days, presumably the minimum period required for a fly to acquire, mature and transmit leishmania parasites (the subject of subsequent chapters). Larger samples and periodical sampling would be necessary in order to obtain more complete information on these aspects of the *Lu. evansi* population dynamics.

# CHAPTER 6

# COLONIZATION AND MORPHOLOGICAL DESCRIPTION

## 6.1 Background

Information on basic aspects of the life cycle such as oviposition, development of immature stages, adult longevity and so on, represent important parameters towards understanding the biology of insect vectors of tropical diseases. In many instances (eg. Culicidae and Reduviidae) aspects of their natural history can be gathered from field observations, however, in other vectors (eg. sandflies), much information is only partly or rarely available from natural conditions. In these cases, colonization in the laboratory represents the most steady and reliable source of information on these insects.

Theoretically, all sandfly species are able to be colonized given appropriate temperature, humidity and space resembling the natural conditions. The high number of sandfly species successfully colonized during the last two decades is testimony to this assertion (Killick-Kendrick et al., 1991; Lawyer et al. 1991). However, some species though reared through at least one generation, have not yet been colonized. Presumably, in these species unknown factors are not mimicked in the laboratory reflect the micro-environment of the species in nature.

The present chapter reports and discusses laboratory studies in addition to field observations on the natural history of *Lutzomyia evansi* in San Andrés de Sotavento. Our final goal was to gain information on the morphological and ecological aspects of this species which might serve as clues on its biotope and to compare it with that of the main vector of visceral leishmaniasis, *Lu. longipalpis*.

## 6.2 Materials and Methods

### 6.2.1 Life cycle

Wild sandflies were collected in Gardenias and El Contento by Shannon trap. Immediately after catching, flies were kept inside Barraud cages with a humid sponge to provide a high relative humidity. Cages were transported to the field laboratory in polystyrene containers and on the same night of capture flies were offered a blood meal from an anaesthetized hamster. Twenty-four hours after feeding, engorged flies were separated and held in cages at 28°C and 90% RH until egg maturation. Cotton pads soaked in an aqueous sucrose solution were provided.

Batches of up to 100 gravid females were put into damp, plastered 300ml Nalgene® pots covered with a mesh cloth which were kept inside polycarbonate boxes (44 x 22 x 37cm). Pots were inspected and fly corpses removed daily up to five or six days post blood-feeding when the majority of females had laid eggs and died. Some pots where kept in the San Andrés field lab but most of them were transported inside polystyrene boxes to CIDEIM headquarters (Cali). In both cases, rearing of the immature stages followed a modified technique described by Modi & Tesh (1983). Briefly, immediately before larval hatching (which is indicated by the presence of caudal bristles and mouth parts visible through the egg shell), a small amount of larval food was sprinkled over the eggs. The food consisted of a matured 1:1 mixture of rabbit faeces and rabbit chow enriched with 5% liver powder. The food was dried and ground through different sieves (obtaining different size of grains to be used for each larval instar). The final product was aliquoted into small vials, sterilized, and kept frozen to avoid acari and fungal growth.

After hatching, larvae were inspected on a daily basis to control humidity, feed the larvae and disrupt any fungi overgrowth and acari infestation. Feeding and moisture were stopped when the majority of the larvae transformed to pupae.

After emerging, adults were counted, sexed and transferred to holding cages with access to sugar solution and water. Four to five day old females were fed on anaesthetized hamsters. In order to identify the females and establish their fecundity and fertility rates, they were left in the cage for two days and then transferred to individual plastered vials (4.0cm x 2.5cm), the bottom of which was lined with filter paper wetted with distilled water. The vials were capped with a cloth mesh, and placed in a polycarbonate boxes. To determine egg development, some females were dissected at 12h intervals. The proportion of mature eggs

(ie. in IV or V stages of Christophers) from bred females was compared with that found in wild caught individuals. The remaining females were allowed to oviposit and when dead, identified and their eggs counted. Eggs from *Lu. evansi* females were pooled into 300ml rearing pots, where the life cycle started again. The duration of various larval instars and their mortality rates were averaged from a variable number of individuals over several generations.

### 6.2.2 Describing immature stages

Egg description was based on recently laid batches of eggs recovered from several pots of $F_3$ laboratory bred females. Eggs were measured using an ocular micrometer attached to an Olympus® compound microscope. A batch of approximately 50 eggs was fixed for 12h in 0.1M cacodylate buffer (pH= 7.2) mixed with 4% glutaraldehyde: 5% paraformaldehyde (Fausto et al., 1991) and transported to London for examination under a scanning electron microscope (SEM). The samples, in a plastic canister, were washed three times with distilled $H_20$ and post-fixed in aqueous 1% $O_sO_4$ for one hour. This was followed by three graded acetone series (15min each), before critical point drying. Specimens were glued with epoxic Araldite® to a SEM stub, gold-coated and observed in a Hitachi® S-450 SEM.

For the morphological description of larval and pupal stages, individuals of different instars were removed from rearing pots, killed in hot distilled water and mounted in Berlese medium for drawing. The morphological description of setation was largely based on Ward (1972) and Foote (1987) rather than Ward (1976).

### 6.2.3 Assessing adult sugar intake, egg batch size and survival

Wild caught flies were kept under lab-field conditions, offering only water on cotton wool for 12h. After this period, flies were blood-fed on hamsters and individually transferred to plastered vials. Flies were evenly distributed between four groups. Each group of 15 individuals was offered one of the following sugar solutions: glucose, sucrose, fructose or maltose, on small cotton wool pads placed on the top of each vial every day. All vials were placed inside clear plastic boxes at 95% RH, 29°C and kept in almost complete darkness for four days. During this interval, disturbance was kept to a minimum, only to change cotton pads and record survival rates. On the fourth day, flies were carefully transferred to damp plaster-lined vials and kept as indicated above for an additional four days after which they were dissected and fecundity estimated from the number of eggs they contained (Tesfa-Yohannes, 1982).

## 6.3 Results

### 6.3.1 Life cycle

A total of five cultures of *Lu. evansi* were obtained under laboratory conditions at 25°C and 89-95% RH in Cali. Two additional cultures were made in SAS (28°C and 99% RH). Data are based on five consecutive generations from each culture, excepting SAS, where only three generations were recorded.

The average time for the total development (ie. between the blood meal intake and the first emergence of adults) of *Lu. evansi* was 41.8 days (range= 35.1-49.6). From this period, the pre-oviposition (ie. blood meal digestion and egg maturation) was 3.5 days (range 2-5) while egg incubation required 6.5days (6.0-7.0). The duration (in days) and respective ranges (in brackets) of the different immature stages are showed in Figure 6.1.

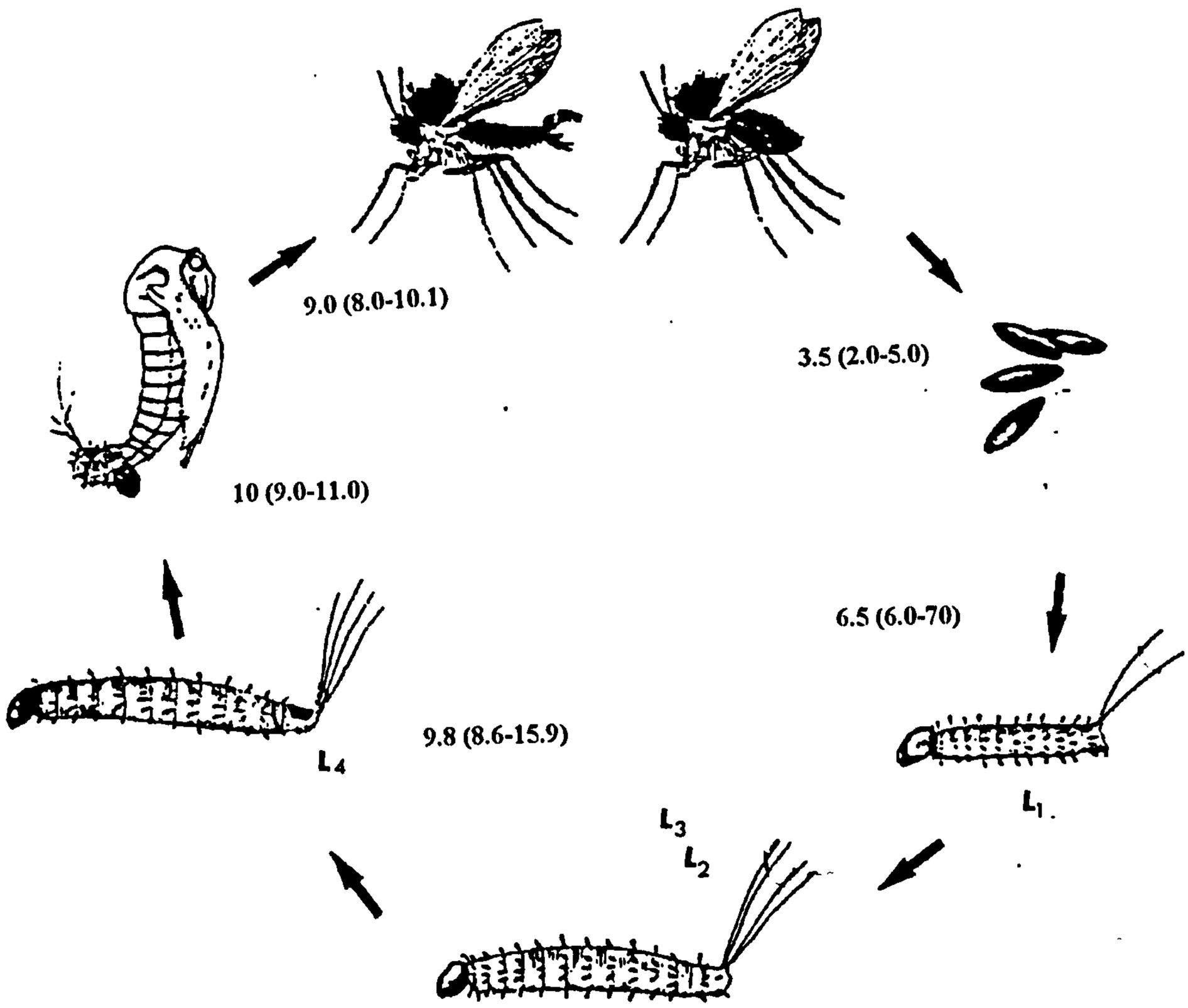



**Figure 6.1** Schematic representation of *Lu. evansi* life cycle

The mean number of eggs laid per female was affected by the type of container used for oviposition. Thus, a lower number of eggs was obtained using pots (13.2 ±4.4; n= 3025♀♀) compared with vials (29.9 ± 3.2; n= 492♀♀), although there was no difference in the fecundity of both groups. The mean number of eggs found in gravid wild caught females was 33.4 (range 10-43; n=140♀♀), although this varied between season (Figure 6.2).

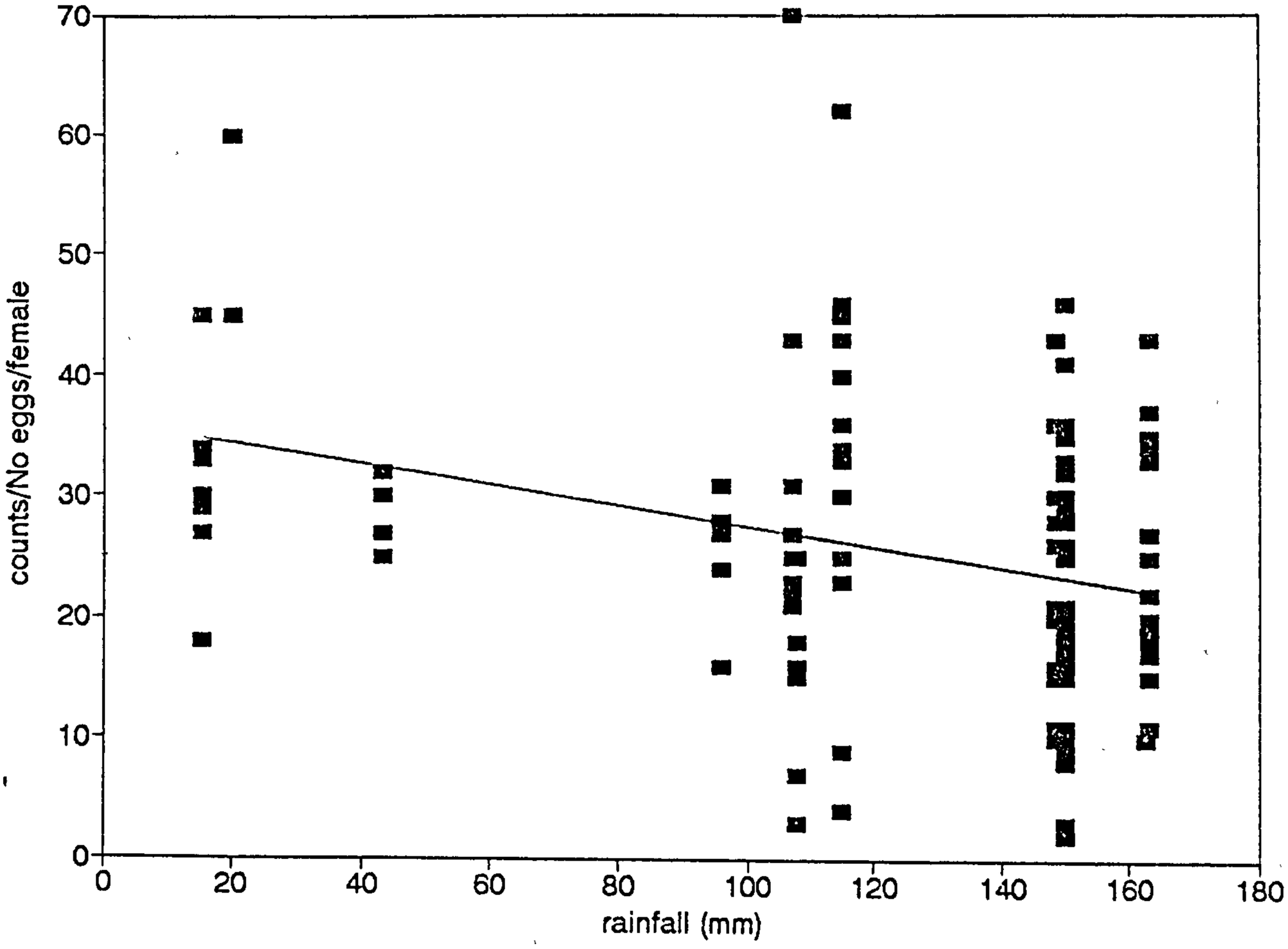



**Figure 6.2** Regression line of the number of eggs found in wild caught *Lu. evansi* (y=36.16- 0.085X; p= < 0.005).

There were no significant differences in larval hatching between CIDEIM and SAS colonies, in both cases being under 50%. Overall larval mortality was 35.6%, from this mortality presumably due to fungal or mite contamination during the first instar represented the highest proportion (Table 6.1). In some instances, proliferation of these contaminants was controlled by removal or disruption with needles and reducing pot humidity. However, the control procedures appeared to affect larval survival too. Mortality at the pupa stage was always low.

**Table 6.1**: Summary of the main events in the colonization of *Lu. evansi* under lab conditions in (A) CIDEIM and (B) San Andrés de Sotavento (Colombia).

| A | ♀♀ | Eggs Laid | Hatched | Mortality | | Adults | | Increase |
|---|---|---|---|---|---|---|---|---|
| | N (x̄/batch) | N (x̄) | Larvae N (%) | $L_1$ | $L_1 \rightarrow L_4$ | ♀♀ | ♂♂ | Rate |
| $P_1$ | 886 | 10904 | 2280 | 570 | 51 | 754 | 997 | ---- |
| | (177.2) | (12.3) | (21.0) | (25.0) | (2.2) | (0.75) | | |
| $F_1$ | 632 | 12599 | 4438 | 479 | 99 | 1819 | 2041 | **0.85** |
| | (126.4) | (19.9) | (35.2) | (10.8) | (2.5) | (0.89) | | |
| $F_2$ | 668 | 9074 | 3979 | 632 | 592 | 1171 | 1584 | **2.41** |
| | (133.6) | (13.6) | (43.9) | (15.9) | (17.7) | (0.73) | | |
| $F_3$ | 849 | 7569 | 2869 | 2322 | 218 | 112 | 104 | **0.64** |
| | (169.8) | (8.9) | (37.9) | (80.1) | (39.9) | (1.07) | | |
| $F_4$ | 31 | 1020 | 405 | 101 | 6 | 112 | 186 | **0.09** |
| | (6.2) | (32.9) | (39.7) | (24.9) | (2.0) | (0.6) | | |
| $F_5$ | 41 | 609 | 262 | 24 | 0 | 123 | 115 | **1.0** |
| | (8.2) | (14.9) | (43.0) | (9.2) | (0) | (1.06) | | |
| T | **3107** | **41775** | **14233** | **4128** | **966** | **4102** | **5027** | |
| | | **(13.4)** | **(34.0)** | **(29.0)** | **(9.6)** | **(0.81)** | | |
| **B** | | | | | | | | |
| $P_1$ | 50 | 3906 | 994 | 460 | 0 | 230 | 156 | ---- |
| | (25.0) | (7812) | (25.5) | (46.3) | (0) | (1.47) | | |
| $F_1$ | 116 | 2442 | 1381 | 130 | 12 | 791 | 448 | **3.43** |
| | (58) | (21.0) | (56.5) | (9.4) | (1.0) | (1.76) | | |
| $F_2$ | 110 | 2114 | 834 | 253 | 0 | 239 | 342 | **0.30** |
| | (55) | (19.2) | (39.5) | (30.3) | (0) | (0.69) | | |
| $F_3$ | 144 | 2825 | 1369 | 199 | 22 | 586 | 562 | **2.45** |
| | (77) | (19.6) | (48.5) | (14.5) | (1.9) | (1.04) | | |
| T | **370** | **11287** | **4578** | **1042** | **34** | **1846** | **1508** | |
| | | **(30.5)** | **(40.6)** | **(22.8)** | **(0.96)** | **(1.22)** | | |

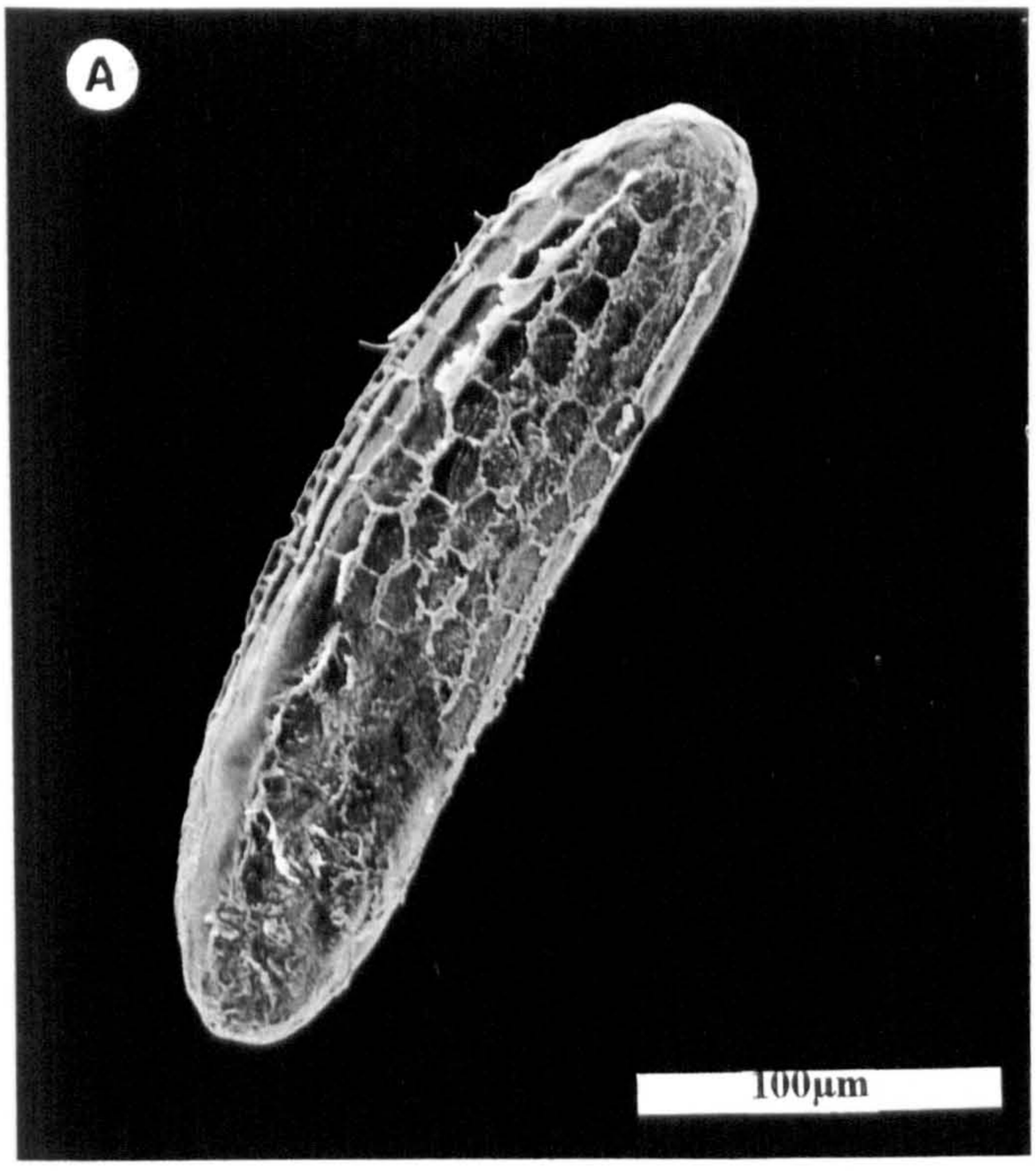
Bred adults were robust and healthy. Generally, females emerged some hours later than males. Overall female:male ratio was 0.90 though it was male biased in individuals grown in pot culture (0.81) in contrast with those in vials (1.15). Blood feeding of adults from the first three generations was easily achieved using either hamster or membrane feeder through a chick-skin. Conversely, females from the fourth and fifth generations fed with reluctance or not at all by these methods. A human arm was accepted by flies but not in all instances.

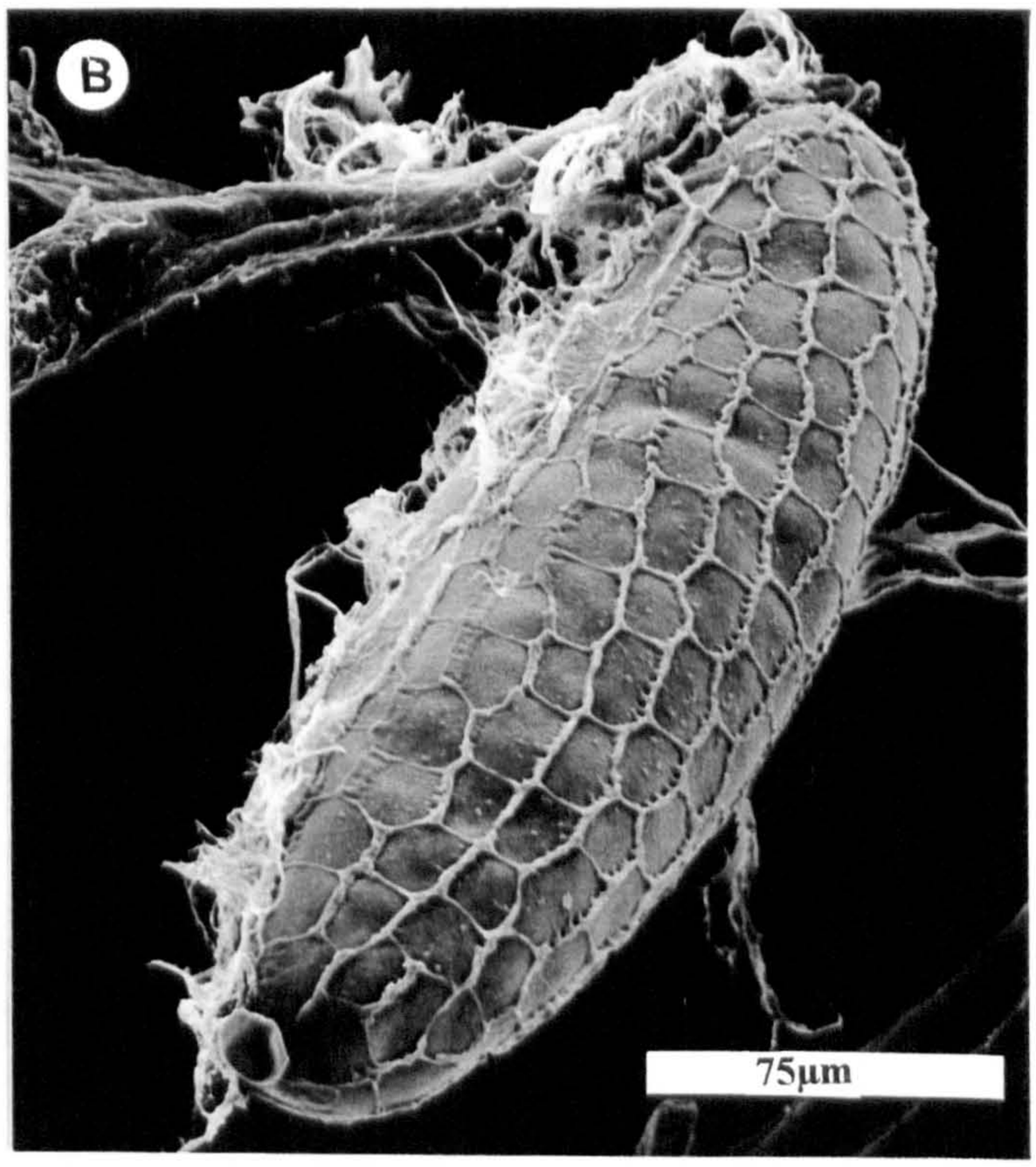
Attempts to colonize *Lu. evansi* under the local conditions of SAS showed more promising results than in Cali: Firstly there was a slight reduction in the length of the cycle (40.3d ± 1.88; range= 37-44); secondly, an increase in the proportion of females (female: male ratio = 1.3) and finally, an increase in the mean number of eggs developed (30.5 eggs ± 14.56; n=370♀♀). On the other hand, there were strong limitations to the colony; in descending order of importance, no hatching and/or death during first larval instar, fungal contamination and ant predation.

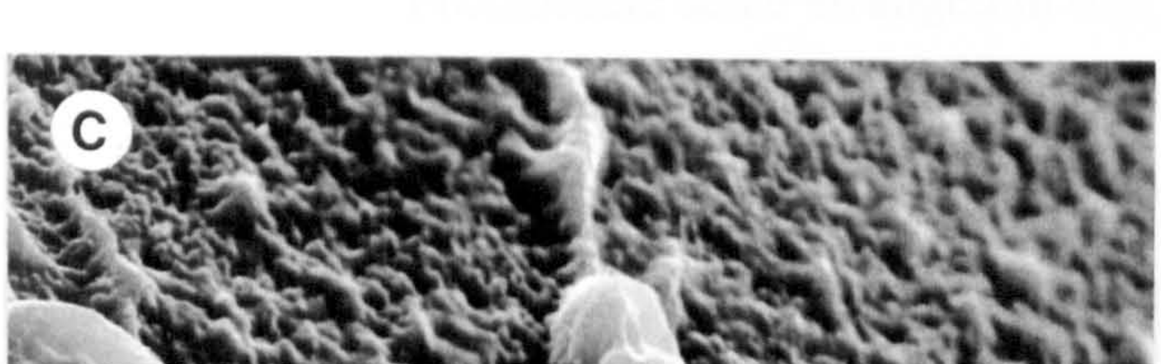
### 6.3.2 Description of the immature stages

6.3.2.1 Egg. The eggs were laid in batches, preferentially on the humid plaster though in occasions lose eggs were observed on the vertical walls of the pots, on the mesh or cotton. Under a light stereoscope, eggs of *Lu. evansi* were dark brown, structurally following the typical ellipsoidal and elongated patterns of the rest of the Phlebotominae. On average they measured 285.6 ± 9.66µm x 8.6 ± 1.08µm (range 250-310µm). Scanning of the outer egg surface (exochorion) reveals the presence of elongated polygons interconnected by bars (Figure 6.3). The main ridges varied in thickness along their length and had small lateral buttresses (C and D, Figure 6.3).

6.3.2.2 Larvae. The newly-hatched larvae were translucid measuring between 750 and 766µm (two specimens). The head capsule was lightly sclerotised, measuring 117µm long and 83µm diameter. As in all other sandflies larvae (except *P. tobbi*) a conspicuous characteristic is the presence of two long, dorsal, caudal setae. A comparison of both length and width of the cephalic capsules between first and second larval stages reveals no dramatic changes between them though, the body was slightly bigger (875-900µm) in the second instar (one specimen). At this stage, the clypeal-labral suture is highly pigmented and the posterior area of the frons has a dark brown spot, from which arises four posterior-antennal (pa) knob-like setae. The



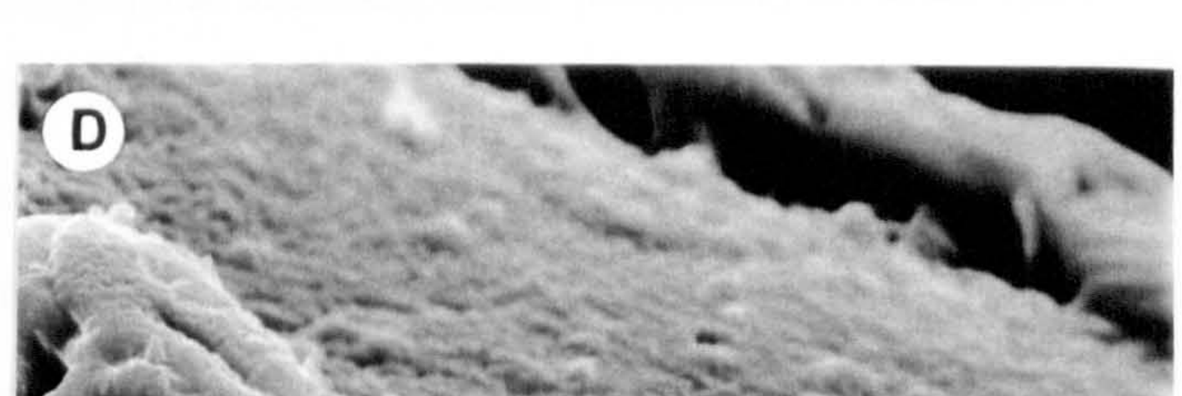




**Figure 6.3** (A and B) Polygonal pattern of outer egg surface of *Lu. evansi* (x300 and x400, respectively). (C and D) Detail of lateral 'buttresses' observed in ridges under high resolution (x7.00K).

dorsal-external prothoracic (d ext p) setae are longer than the internal prothoracic (d int p) and the remaining meso and metathoracic setae are not well developed. An important feature is the presence of two pairs of caudal setae.

Fourth instar (3 specimens) larva over four or five times length($\bar{x}$= 3.2mm) and width of first instar. Figure 6.4 shows the main morphological and setal characteristics of a typical fourth instar larva. Head completely sclerotised and pigmented, 300µm long and 200µm wide. Antennae with two segments, totalling 33µm in length, proximal segment slightly longer than distal. Mouth parts with four teeth and, a mento (clypeus) with four tooth-like projections. Clypeal setae (c) simple, spine-like and forward directed. Anterior-dorsal (ad) setae spine-like, 58 µm, nearly double length of antenna. Two posterior-dorsal setae (pd) thicker than anterior-dorsal setae, inserted near antenna. Four knob-like posterior-antennal setae (pa), two near epicranial suture and two near genae. All posterior-antennal setae arranged in a transverse line and equidistantly separated on the dorsal-lateral region of head. Lateral genal setae (lg) 41µm, antero-ventral to lateral setae ( l ). Ventral genal setae very short (25µm), spine-like, near labium.

Prothoracic setae arranged in two rings (one anterior, other posterior) all knob type and of different lengths. Dorsal internal prothoracic setae (d int p) 100µm, external (d ext p) 50 µm. The dorsal-lateral setae (one on each side) 100µm, anterior ventro-lateral setae (avl) 75µm. Prothoracic spiracle present between dl and avl setae. Ventrally, internal (v int p) and external (v ext p) ventral prothoracic setae 75µm long, distally touching labium and genae. Internal posterior dorsal setae (ipd) 108µm, remaining setae shorter: dorsal submedian (dsb), 47µm and dorso- lateral setae (dl), 67µm. Ventrally, two setae given their proximity to anterior ventrolateral (avl) setae, are probably homologous to mesothoracic basal (b) setae followed by posterior ventrolateral setae (pvl), 67µm long and four ventral submedian setae, two long (25µm), two very short (8.3µm).

Dorsal submedian (dsb) and internal posterior dorsal (ipd) most prominent setae in meso-metathorax area, at length of 117µm. Laterally, in a transverse line: dorsolateral (dl) (100µm) and anterior ventrolateral (avd) setae (75µm). Ventrally posterior ventrolateral (pvl) and ventral submedian (vs) setae, same length (50µm). Short setae (15µm) centrally positioned are probably ventral submedian basal (vsb) setae.

Abdomen: From the first to 7th abdominal segments all setae knob-like, setal map almost identical on all segments, with some exceptions. Dorsal submedian (dsb) setae 150µm long on first two abdominal segments but decreasing from third (67µm) until total disappearance

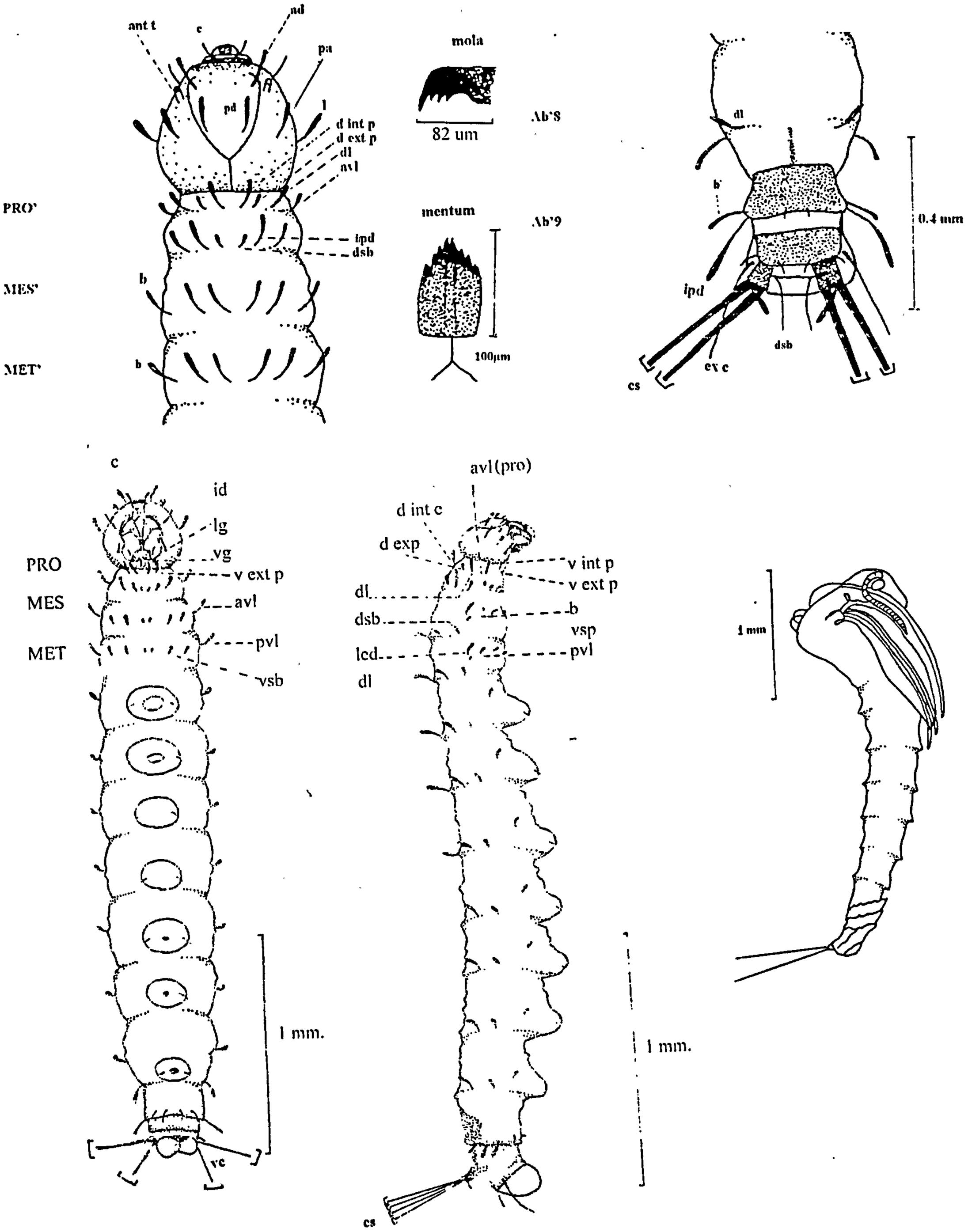



**Figure 6.4** Immature stages of *Lu. evansi*. Top half: setal map of head, thorax, and last abdominal segments of 4th instar larvae (dorsal view) and detail of mola and mentum. Bottom half: Ventral and lateral view of 4th instar and pupa (lateral).

at 6th-9th segments. Internal posterodorsal (ipd) setae elongated, 83µm long on all segments. Same length as basal (b) and dorsal submedian (dsb) setae. Anterior ventral (avl) setae in anterior part each segment, smaller than basal setae (66µm).

Ventrally, postero-ventral (pvl) and ventral submedian (vsb) setae unseen on 1st to 7th segments. 'Feet' (cf. Ward, 1972) elongated, distally ending in rounded suction-like structure. Two small spine-like setae on each side. Some individuals pigmented on posterior segments.

Dorsally, second and third annuli in 8th segment strongly pigmented. Knob-like setae less obvious. Dorsal submedian (dsb) setae, present but very short. Internal postero-dorsal (ipd) setae very long (117µm). Ventrally, basal (b) setae shorter (83µm) and thinner than other setae. Remaining ventral setae (vsb, pv, pvl and ext a) spine-like, no longer than 25µm.

Caudal segment (9th) heavily pigmented with two lateral projections, each with paired setae. External pair 950µm long, interior pair, 767µm. Ventrally, ventral caudal (vc) setae (33µm). Dorsally, external caudal (ex c) setae present and posteriorly a pair of spine-like setae probably, internal posterior anal (I post a) setae, outer 183µm long, inner 91.5µm extending beyond 'anal foot'.

6.3.2.3 Pupae: 2.28mm long (two specimens). Dorsal width in wing insertion point 0.5mm; prealar setae simple, spine-like, 133µm, usually reaching mesonotal protuberance.

### 6.3.3 Assessing adult sugar intake, egg batch size and relative survival

The cumulative mortality at 48h was significantly higher ($p<0.05$) in flies fed on maltose and glucose than either fructose and sucrose (Figure 6.5); all flies died in all groups, the majority of them immediately after oviposition when a wet surface was provided. However, the former pair (maltose and glucose) died after six days whilst some flies fed on fructose survived to eight days. (Figure 6.5).

Similarly, the size of egg batches was also affected by the type of sugar provided. Flies fed on sucrose or fructose laid more eggs than those fed on maltose or glucose ($p<0.05$). This assumes that the variation in blood-meal size was the same in each sugar-group, ie. there was no systematic bias which could have caused the differences in the number of eggs produced in each group.

Figure 6.3
Effect of sugars source
on Lu. evansi survival

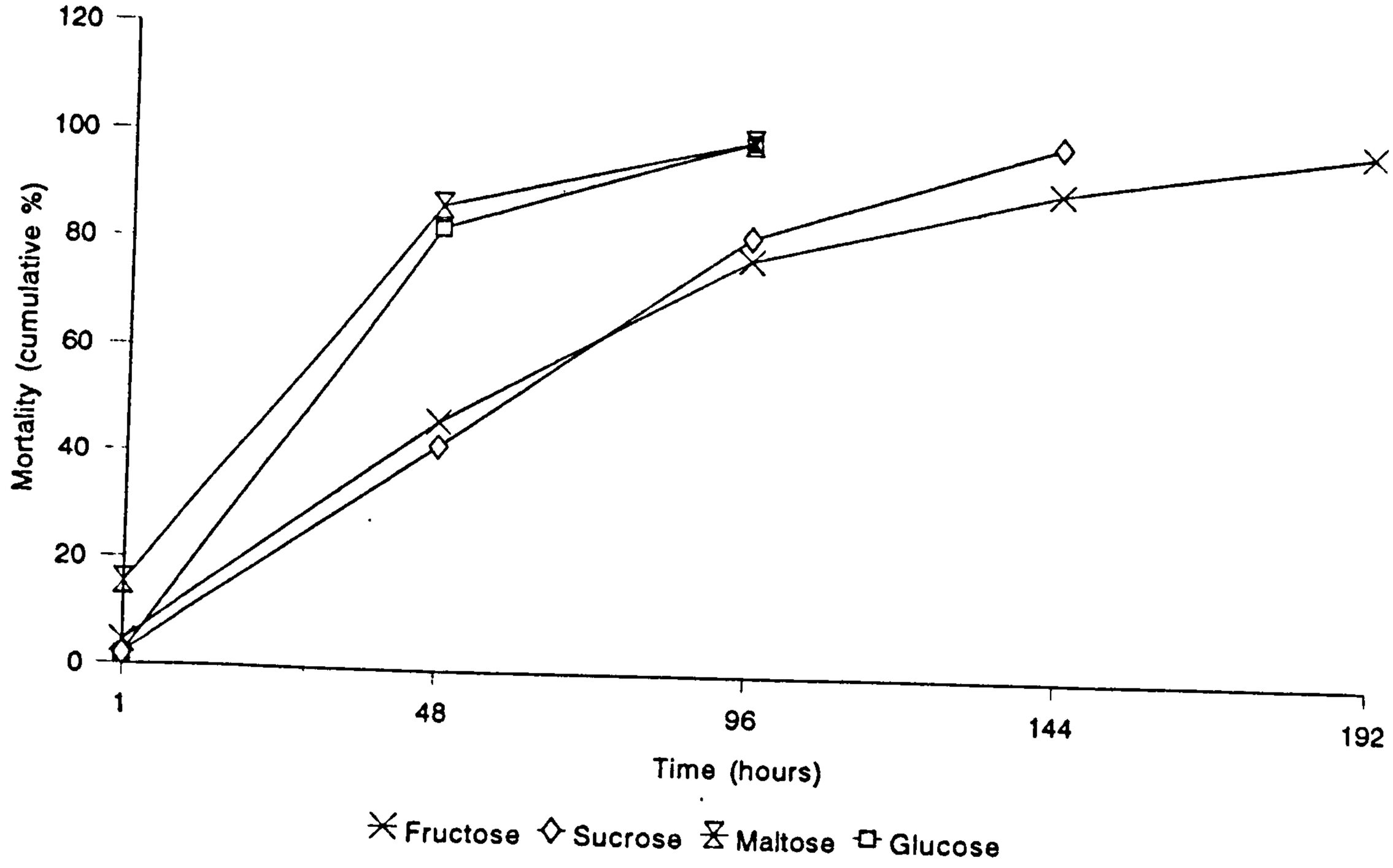



**Figure 6.5** Effect of sugar source on *Lu. evansi* survival

## 6.4 Discussion

The duration of the entire life cycle of *Lu. evansi* reported here confirms the preliminary observations of Mirsa (1953), who found that over a range of temperatures and relative humidity, this species had a development time between 31 and 51 days. Our results are also consistent with the data recently reported by Oviedo et al. (1995) who colonized Venezuelan *Lu. evansi* at 26°C and 90% RH.

**Table 6.2** Duration* of different instars in various attempts to colonise *Lu. evansi* in Colombia (SAS[1] and Cali[2]) and Venezuela (Trujillo,[3] Oviedo et al., 1995; and Altagracia,[4] Mirsa, 1953).

| Strain | E→$L_1$ | $L_1$→$L_2$ | $L_2$→$L_3$ | $L_3$→$L_4$ | $L_4$→P | P→A | Total |
|---|---|---|---|---|---|---|---|
| **Colombia** | | | | | | | |
| SAS | 6.5 | 6.1 | 3.8 | 6.0 | 10.0 | 9.0 | 41.4 |
| Cideim | 6.0 | 7.0 | 6.0 | 6.0 | 10.0 | 9.0 | 44.0 |
| **Venezuela** | | | | | | | |
| Trujillo | 6.8 | 7.2 | 4.0 | 6.5 | 7.9 | 8.1 | 40.5 |
| Altagraci | 10 | 9.0 | 9.0 | 9.0 | 9.0 | 5.0 | 51.0 |

[1] 28° C- 90% RH; [2] 25°C- 90% RH ; [3] 26°C- 90% RH ; [4] 28.6°C- 90% RH
* (in days)

Despite our successful colonisation of other sandfly species (*Lu. longipalpis*, *Lu. gomezi*, *Lu. lichyi*) in the laboratory in Cali, we could not culture *Lu. evansi* beyond the fifth generation. In addition to the inherent problems associated with colonisation of wild caught specimens (eg. female egg retention, larval loses due to fungus or mite contamination, etc.) the main challenge to our colony was the reluctance of females of the fifth generation to feed on the blood sources provided. Even when some did feed and eggs developed, they did not oviposit. Our experience is in strikingly contrast to Oviedo et al. (1995) who claimed a high productivity from their colony, currently in its 9th consecutive generation. Interestingly, M. Oviedo (per. comm) experienced a similar "bottle-neck" in the colonization of *Lu. evansi* but unfortunately no explanation was given on how the problem was overcome. Since no obvious differences were found when we compared our own rearing methods, we suspect that *Lu. evansi* is a stenogamic species.

The life cycle of *Lu. evansi* in the laboratory appears to be similar to that recorded in other colonized sandfly vectors viz. *Lu. longipalpis* (Killick-Kendrick et al., 1977); *Lu. intermedia* (Rangel et al., 1985); *Lu. whitmani* (Barretto, 1941), *Ph. papatasi* (Modi & Tesh, 1983; Pandya, 1980) and *Ph. argentipes* (Ghosh & Battacharya, 1989). We suspect that the Venezuelan species has more adaptability to lab conditions than the Colombian counterpart. Such variation in adaptability between different populations of the same species is not a rare phenomenon in sandflies, rather it has been observed by several workers in their attempts to colonise both Old and New World species (Dr. R Lane, pers. comm.).

Another aspect which needs to be solved for the successful colonization of *Lu. evansi*

is related to egg retention by females. Results of dissection of individually kept females from the controlled experiments using different sugars, revealed that fully blood-fed female *Lu. evansi* can develop up to 60 eggs/batch, contrasting greatly with the mean eggs laid per female in our cultures (potted females = 11.63; vialled females = 32.57). It is important to note that the mean number of eggs found in wild females caught by sticky traps over nine months was similar to that observed in laboratory bred females. This is highly suggestive that in nature there is a seasonal variation associated with the environmental conditions. Thus, the drier the environmental conditions the bigger of batch of eggs developed by *Lu. evansi* females (Figure 6.2).

Externally, the egg morphology of *Lu. evansi* resembles that described by Feliciangeli et al . (1993) for Venezuelan species of the *Verrucarum* group (*Lu. evansi*, *Lu. townsendi*, *Lu. youngi* and *Lu. spinicrassa*). All of them exhibit a polygonal pattern, which actually is widely shared by several other New World sandfly species (cf. *Lu. sanguinaria*, *Lu.gomezi*, *Lu. trapidoi* and *Lu. ylephiletor* (Endris et al., 1987) and those of the *intermedia* group (Ward & Ready 1975). However, variations in the sculpture, distribution and size of the exochorionic ridges show differences at specific or group levels. For instance, an elongated polygonal pattern distinguishes the eggs of *Lu. evansi* from *Lu. youngi*, *Lu. verrucarum* and *Lu. spinicrassa* which have a pentagonal, hexagonal and irregular geometric pattern, respectively (Feliciangeli et al., 1993). In addition, the buttress lobules (Figure 6.3D) present in *Lu. evansi* appears to be unique in *Lutzomyia* to our knowledge. In the absence of more detailed information is difficult to make any inference about the real ecological significance of all these patterns but any plausible explanation is likely to depend on specific adaptation to micro-environments. In this regard it is important to point out that the eggs of two of the most adapted peridomiciliar *Leishmania* vectors, *Lu. longipalpis* and *Lu. intermedia*, present a quite uncommon unconnected ridges pattern which strongly contrast with the majority of known vectors which mainly have a polygonal pattern.

The larval stages of *Lu. evansi* parallel with those described by Hanson (1968) for *Lu. serrana* and *Lu. ovallesi* though they are more similar to *Lu. ovallesi*. Despite the huge importance paid to adult stages of *Lu. longipalpis* very little attention has been given to its immature stages. Actually the only work on this regard was done by Guitton & Sherlock in 1969. Although these authors produced an excellent number of graphic illustrations, unfortunately they made little effort to provide measurements of the different setae, making

any meaningful comparison of their work with ours difficult.

The importance of sugar (sucrose) intake for egg production in *Lu. longipalpis* was demonstrated by Ready (1979) but there have not been any studies on the relative importance of different sugars. Besides finding a similar effect in *Lu. evansi*, our results also suggested that fructose increased female longevity and vitellogenesis in *Lu. evansi*.

# CHAPTER 7

# NATURAL AND EXPERIMENTAL INFECTIONS

## 7.1 Background

One of the most pertinent steps in determining the competence of any sandfly species in *Leishmania* transmission is the search for sandflies infected with *Leishmania* in nature or alternatively, their experimental susceptibility to infection by this parasite. The quantitative expressions of the ability to harbour parasites are referred as the natural and experimental infection rates, respectively. Epidemiologically, the former has more relevance than the second. However, since infection rates in nature are generally very low (< 1%) searching for natural infections by individually dissecting sandflies is a difficult, time-consuming and, sometimes, expensive process. For these reasons, experimental infection is a reasonable alternative for estimating susceptibility and transmission potential of the suspected vectors in an area. During the last two decades, the establishment of sandfly colonies has enabled a variety of experimental assays to be developed, mainly directed at the study of the life cycle of *Leishmania* and to explore the associations between these trypanosomatids and their vectors. Important stages in the sequence of parasite development in the sandfly gut such as colonization, differentiation, migration and attachment has been elucidated as well as sandfly susceptibility, refractoriness and transmission capacity by testing natural and unnatural parasite/vector combinations (Killick-Kendrick, 1985; Walters, 1993). In the New World, pioneering work, though rather fragmentary, has provided complete ultrastructural examinations on the development of *Leishmania* species in some sandfly species. An invaluable documentation has been gained from the studies on natural or likely associations, such as *L. chagasi/Lutzomyia longipalpis* (Lainson et al., 1977; Walters et al., 1989b; Elnaiem et al., 1992; 1994); *L. mexicana* with *Lu. pessoana* (Strangways-Dixon & Lainson, 1966), *Lu. diabolica* and *Lu. shannoni* (Lawyer et al., 1987) and *L. panamensis/Lu. gomezi* (Walters et al., 1989a) as well as in non-natural associations, like *L. mexicana/Lu. abonnenci* (Walters et al., 1987) and *L. panamensis/Phlebotomus papatasi* (Walters et al.,

1992). Overall, results indicate a complete development of leishmaniae in the gut of co-evolved sandfly species but, on the other hand, variable degrees of refractoriness in non-related flies (Killick-Kendrick, 1985; Walters, 1993).

Classical studies on the epidemiology of American visceral leishmaniasis due to *L. chagasi*, postulated an association of this parasite with the sandfly *Lu. longipalpis*. This assumption, mainly based on the correlation of human cases with the geographic distribution of *Lu. longipalpis* (Chagas et al., 1938; Grimaldi et al., 1989), has received substantial support from the finding of naturally infected flies and experimental and ultrastructural microscopy studies (Lainson et al., 1977; Walters et al., 1989b; Elnaiem et al., 1992; 1994). All of these have reiterated the statement that *Lu. longipalpis* is the only vector of *L. chagasi* throughout its geographical range. Nevertheless, there are areas in Colombia, Venezuela and Brazil where AVL is endemic but, the presence of *Lu. longipalpis* has not been recorded (Travi et al., 1990; Blanco-Tuirán et al., 1993; Travi et al., in press; Moreno & Oviedo 1995; Cat et al., 1974; Jeronimo et al., 1994), which raises the issue of whether *L. chagasi* can be transmitted by alternate vectors. The finding of *Lu. evansi* naturally infected with *L. chagasi* in the San Andrés de Sotavento focus, an area free of *Lu. longipalpis* (Travi et al., 1990; Travi et al., in press) prompted the present study[1], which firstly examines the natural infection rates of the most abundant sandfly species in the area, while in the second part compares the development of two strains of *L. chagasi* in both its dominant vector (*Lu. longipalpis*) as well in its occasional vector (*Lu. evansi*).

## 7.2 Methods

### 7.2.1 Natural infection rates

Female *Lutzomyia* obtained by man-biting, Shannon and animal baited traps, and direct aspiration captures from resting places were individually dissected, their midgut examined for natural infection and, when possible, the parous status determined (Ready et al., 1984). For dissection, flies were handled following the technique of Johnson et al. (1963) with modifications. Thus, flies were washed with a weak detergent solution to remove as much contaminant material as possible before transfer to 10% PBS + penicillin/streptomycin

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1 Part of the information contained in this chapter was presented in a 15min session at the Royal Society of Tropical Medicine (Manson House) on November 16th 1995. Abstract of the talk is included in the appendix 7.1

solution. Two drops of this solution were put on a clean, autoclaved slide. Wings and legs of the specimen were removed in the first drop and the rest of the body transferred to the second. After making a short incision at the penultimate tergite, the head was slowly pulled out together with the entire fore and midgut. The preparation was covered with a sterile coverslip and examined by light microscope. The gut contents of all flies positive or suspected of harbouring flagellate forms were aseptically inoculated part into culture tubes containing Senekie's blood-agar medium plus streptomycin-penicillin-fluorocytosine and the remainder into a hamster via intraperitoneal inoculation. Tubes were examined daily for two weeks. Inoculated hamsters were tagged and followed for up to 11 months for clinical manifestations of disease. At the end of this period, survivors were killed and immediately after dissected. Heart blood was drawn and cultured in addition to macerates from liver and spleen tissues. Plate smears of liver and spleen were stained with Giemsa and examined under contrast phase microscopy.

### 7.2.2 Experimental infections

The following experiments were carried out using two strains of *L. chagasi*. The first strain, L-12 (MHOM/COL/90/L12), was isolated in 1990 from a two-year-old child in San Andrés de Sotavento (SAS). The second, Nilo (MCAN/COL/95/NILO), was isolated from a dog native to El Nilo (Cundinamarca), a locality situated in the AVL endemic area of the Magdalena valley where *Lu. longipalpis* is the only known vector (Corredor et al., 1980; Morrison, 1994).

Wild-caught *Lu. evansi* from SAS and a laboratory bred Colombian strain of *Lu.* longipalpis originally from the El Callejón (Magdalena Valley), were employed in these experiments. Wild caught *Lu. evansi* were caught in Shannon traps between 19:00- 21:00h, and used in experiments the same night. Prior to and after infection, caged flies were placed in plastic bags and kept in the shade outside the field laboratory at a mean of 28°C (range 18-32°C) and over 90% relative humidity. Cotton pads soaked in an aqueous sucrose solution and water were provided every day.

Unless otherwise stated, all experimental infections and dissection of flies was carried out in a simple field lab' in SAS to avoid transporting flies to the main lab' in Cali.

Sandflies were infected either directly on infected hamsters or indirectly (via an artificial membrane feeder). Beforehand, in Cali, parasites were grown in Senekie agar-blood medium and juvenile hamsters (*Cricetus aureatus*) were inoculated intraperitoneally with

cultured promastigotes.

7.2.2.1 Infection from hamsters (Experiment 1): Batches of *Lu. evansi* and *Lu. longipalpis* (in separate Barraud's cages), were fed on L-12 infected hamsters. A pair of hamsters were anaesthetized with 1:10 Ketamine-Rompun®, wrapped in aluminium foil but leaving uncovered the ear and belly areas, and then put inside cages in darkness. Flies were allowed to feed **ad libitum** for approximately 20min. The same procedure was repeated using hamsters infected with the Nilo strain. Three replicates were carried out for each parasite-sandfly species combination.

7.2.2.2 Infection with promastigotes (Experiment 2) : Promastigotes from culture tubes were harvested by centrifugation and washed twice in PBS (pH 7.4). The number of parasites was estimated by counting them in a Neubabuer chamber and then added to one ml of inactivated/defibrinated rabbit blood so the final concentration was 1.0 x $10^6$ promastigotes/ml. Since initially poor infectivity rates were obtained with this parasite dosage with either L-12 or the Nilo strain, it was increased to 2 x $10^6$ parasites/ml in subsequent experiments. Results recorded here correspond only to assays using the higher parasite dosage which was offered to separately caged *Lu. evansi* and *Lu. longipalpis* through a chicken-skin membrane feeder. Wherever possible, the membrane was freshly prepared, tied and trimmed over the open end of a vial tube (2.0 x 4.5 cm) and the feeder vials partially introduced into each cage, tightly tied to the sleeve by rubber bands and left for 20-60min. At 10min intervals the feeder's contents were slightly agitated. Immediately after feeding, a drop of infected blood was examined to assess parasite motility and hence viability. The same procedure was independently repeated for each parasite strain. Three replicates of each parasite-sandfly species combination were made, one replicate each in January and May/93 and September/94.

7.2.2.3 Infection with amastigotes (Experiment 3): Amastigote homogenates were obtained from the spleens of hamsters 3-8 months post-infection. Infected spleens were macerated in one ml of PBS, twice washed with PBS, centrifuged (2,500 rpm) and, finally, the pellet resuspended in 2.0ml of heat-inactivated rabbit blood. The final suspension was poured into two chicken-skin membrane feeders and offered to the sandflies as in the former experiment.

Also, the same procedure was followed but this time using nose homogenates from recently infected hamsters (ie. five-day post-infection in the nose with 1.0ml of 1 x $10^6$

promastigotes/ml of Nilo strain). In this instance, the homogenate was used to feed only batches of *Lu. evansi*. As a control, females of the same species, were fed on uninfected defibrinated/inactivated blood. In both, spleen and nose assays, amastigote presence was assessed by examination of stained tissue smears.

### 7.2.3 Assessing infection and parasite development

At intervals from 24h to 8 days post feeding, batches of flies were dissected and inspected for parasite presence and development in their guts. Forms of parasites were described according to Walter's terminology (1993). Infections in different parts of the alimentary canal were recorded and scored on a $\log_{10}$ scale according to Warburg **et al.** (1991).

### 7.2.4 Assessing parasite transmissibility:

This was carried out using flies 7 to 8 day post-feeding from experiments 2 and 3. Batches of putatively infected flies were offered clean juvenile hamsters. These re-fed flies were dissected and inspected for parasites. The finding of at least one infected fly per batch was considered adequate for follow up of the exposed hamster; these were tagged and followed up for 10-11 months for any symptoms compatible with AVL disease. Unfortunately the hamsters died before they could be biopsied. Therefore, a second approach was used in which the transmission of parasite was simulated by forcing flies to "feed" on capillary tubes containing PBS, following Hertig & McConnell's technique (1963). The contents of each capillary were subsequently smeared on a slide, stained and inspected by light microscope.

### 7.2.5 Survival and fertility rates

Dead flies were removed daily and dissected for the presence of flagellates. Mortality rates recorded on 0 and 5 days post-feeding intervals were compared by Chi-square test in both sandfly species. Additionally, the number of developing eggs (ie. in Christopher's stage IV and V) in infected and uninfected *Lu. evansi* flies were counted at death or when all flies were killed at 6-8 days post infection.

## 7.3 Results

### 7.3.1 Natural infection rates

A total of 5,832 female flies from eight species were individually dissected. From these, only five of 229 *Lu. cayennensis* (2.18%) and three of 5,326 *Lu. evansi* (0.06%) were found harbouring leishmanial forms in their guts. In all positive *Lu. cayennensis*, parasites were easily seen with the light microscope. They were very long and slender promastigotes, forming very heavy masses of motile individuals restricted to the ileum, rectum and in lesser numbers in the pylorus and Malphigian tubules. All positive flies were collected in resting places: three in March and two in July. Attempts were made to culture parasites in Senekie and Schneider media, but none grew successfully. On one occasion, parasites were given orally to two specimens of the red-headed lizard (*Anolis* sp.), which were bled for three days; however, no recovery of parasites was achieved either.

Two of the natural infections detected in *Lu. evansi* came from specimens collected on human bait and in a Shannon trap in July and November/93, respectively. In both cases it was necessary to cover the preparations with a coverslip to see the infections. The predominant forms were nectomonads, which were very motile and mainly detected in the abdominal and thoracic (stomodeal valve) regions of the midgut of the flies, though small rounded or oval bodies were observed lying there, too. The third infected fly was found in January/93. It was a wild caught fly used for an experimental infection assay with *L. chagasi*. Since parasites from the experimental infection were still encapsulated by the peritrophic membrane it was assumed that the promastigote infection present in the pylorus and ileum corresponded to a natural infection. The parasitemia was heaviest in the pylorus and paramastigotes were the dominant forms.

### 7.3.2 Experimental infection rates

The total number of exposed flies, feeding rates and infection frequencies, in each experiment are summarized in Tables 7.1 and 7.2

7.3.2.1 Experiment 1:Infection from hamsters. Although a considerable number of both *Lu. evansi* and *Lu. longipalpis* were fed on infected hamsters, no infection was recorded with either *L. chagasi* strain (Table 7.1). Dissections of blood-fed flies at four and five-day post feeding revealed no detectable changes in the sandfly midgut while digesting the blood meal.

**Table 7.1.** Totals of *Lu. evansi* and *Lu. longipalpis* exposed (N) and percentages fed and infected (infec) using infected hamsters (Exp.1); promastigotes (Exp. 2) or amastigotes (Exp. 3) of two strains of *L. chagasi.*

| | *Lutzomyia evansi* | | | | | | *Lutzomyia longipalpis* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L-12 | | | Nilo | | | L-12 | | | Nilo | | |
| | N | Fed | Infec | N | Fed | Infec | N | Fed | Infec | N | Fed | Infec |
| Exp 1 | | | | | | | | | | | | |
| R-1* | 44 | 25 | 0 | 19 | 12 | 0 | 40 | 29 | 0 | 38 | 31 | 0 |
| R-2 | 72 | 65 | 0 | 61 | 31 | 0 | 50 | 41 | 0 | 71 | 59 | 0 |
| R-3* | 180 | 145 | 0 | 190 | 117 | 0 | 120 | 80 | 0 | 96 | 70 | 0 |
| TOTAL | 296 | 235 | 0 | 270 | 160 | 0 | 210 | 150 | 0 | 205 | 160 | 0 |
| Mean | 98.7 | 78.3 | 0 | 90 | 53.3 | 0 | 70 | 50 | 0 | 68.3 | 53.3 | 0 |
| Exp 2 | | | | | | | | | | | | |
| R-1 | 97 | 69 | 0 | 89 | 66 | 13.6 | 80 | 31 | 2.5 | 80 | 59 | 35.6 |
| R-2* | 50 | 29 | 0 | 230 | 165 | 8.5 | 170 | 129 | 6.5 | 80 | 70 | 17.1 |
| R-3 | 93 | 73 | 0 | 120 | 78 | 10.2 | 120 | 79 | 2.5 | 85 | 60 | 20.0 |
| TOTAL | 240 | 171 | 0 | 439 | 309 | 10.3 | 370 | 239 | 6.3 | 245 | 189 | 23.8 |
| Mean | 80 | 57 | 0 | 146 | 103 | 10.7 | 123 | 79.6 | 3.8 | 81.7 | 63 | 24.2 |

**Table 7.1** (Contd..). Totals of *Lu. evansi* and *Lu. longipalpis* exposed (N) and percentages of fed and infected (infec) using infected hamsters (Exp.1); promastigotes (Exp. 2) or amastigotes (Exp. 3) of two strains of *L. chagasi*

| | *Lutzomyia evansi* | | | | | | *Lutzomyia longipalpis* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L-12 | | | Nilo | | | L-12 | | | Nilo | | |
| | N | Fed | Infec | N | Fed | Infec | N | Fed | Infec | N | Fed | Infec |
| Exp 3 | | | | | | | | | | | | |
| R-1 | 80 | 55 | 0 | 80 | 40 | 2.5 | 82 | 62 | 0 | 60 | 31 | 3.2 |
| R-2 | 92 | 40 | 15.0 | 53 | 38 | 10.5 | 70 | 50 | 0 | 45 | 24 | 8.3 |
| R-3 | 77 | 33 | 6 | 120 | 74 | 5.4 | 86 | 47 | 25.5 | nd | nd | nd |
| TOTAL | 249 | 128 | 6.3 | 253 | 152 | 5.9 | 238 | 159 | 7.5 | 105 | 55 | 5.5 |
| Mean | 82.7 | 42.6 | 7.0 | 84.3 | 50.6 | 6.1 | 79.3 | 53 | 8.5 | 52.5 | 27.5 | 5.8 |

* replicates performed in Cali simulating similar temperature and relative humidity conditions to those in SAS.

**Table 7.2A**. Development of *Leishmania chagasi* (L-12 and Nilo strains) infections in wild-caught *Lutzomyia evansi*.

| | | % (N) infected flies | | | | | |
|---|---|---|---|---|---|---|---|
| | TOTAL FED | Infected | Head | Stomod. valve | Ant. Midgut | Post. Midgut | Hindgut |
| **L-12 strain** | | | | | | | |
| Experiment 1 | 235 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment 2 | 171 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment 3 | 128 | 8 | 0 | 0 | 3 (38) | 8 (100) | 5 (63) |
| **Nilo strain** | | | | | | | |
| Experiment 1 | 160 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment 2 | 309 | 31 | 0 | 2 (7) | 25 (81) | 31 (100) | 8 (26) |
| Exp. 3 (spleen) | 152 | 9 | 0 | 0 | 5 (56) | 9 (100) | 2 (22) |
| Exp. 3 (nose) | 69 | 2 | 0 | 0 | 1 (50) | 2 (100) | |
| TOTAL | 1177 | 50 | 0 | 2 (4) | 34 (68) | 49 (98) | 15 (30) |

**Table 7.2B.** Development of *Leishmania chagasi* (L-12 and Nilo strains) infections *Lu. longipalpis* 1-8 days post-feeding.

| | | | N (%) infected flies | | | | |
|---|---|---|---|---|---|---|---|
| | **Total Fed** | Infected | **Head** | **Stomod. valve** | **Ant. Midgut** | **Post. Midgut** | **Hindgut** |
| **L-12 strain** | | | | | | | |
| Experiment 1 | 150 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment 2 | 239 | 15 | 0 | 0 | 8 (53) | 14 (93) | 0 |
| Experiment 3 | 159 | 12 | 0 | 2 (17) | 5 (42) | 11 (92) | 0 |
| Nilo strain | | | | | | | |
| Experiment 1 | 160 | 0 | 0 | 0 | 0 | 0 | 0 |
| Experiment 2 | 189 | 45 | 0 | 4 (8.9) | 37 (82) | 43 (96) | 0 |
| Experiment 3 | 55 | 3 | 0 | 1 (33) | 3 (100) | 3 (100) | 0 |
| TOTAL | 952 | 75 | 0 | 7 (9) | 53 (71) | 71 (95) | 0 |

Additionally, no alterations in sandfly behaviour and survival rate were observed. It is important to point out that it was confirmed parasitologically all the hamsters used were infected.

7.3.2.2 Experiment 2: Infection with promastigotes. Variable percentages of infectivity and development of *L. chagasi* were documented in all experimentally infected *Lu. evansi* and *Lu. longipalpis* using cultured promastigotes.

Considerable variations were found between infection rates of *Lu. longipalpis* and *Lu. evansi* using the two *L. chagasi* strains (Table 7.1). Overall L-12 parasite infection rates were lower compared with the Nilo strain. In the *Lu. evansi* group, none of the 171 blood-fed flies became infected with L-12. The same strain showed a relatively low infection rate (on average 6.3%) in *Lu. longipalpis*, though in this vector the parasites appeared to be physiologically aberrant, sometimes generating flagellate-like forms difficult to discern. On the other hand, Nilo promastigote forms underwent a consistent development pattern within both the *Lu. evansi* and *Lu. longipalpis* replicates, the latter displaying significantly higher infection rates ($\chi^2$= 28.06; p= 0.000).

7.3.2.3 Experiment3:Infection with amastigotes. Spleen homogenates rendered very low sandfly infection rates. No significant differences were found between *Lu. evansi* and *Lu. longipalpis* infection rates, using either L-12 ($\chi^2$= 0.04; p= 0.84) or Nilo (Fisher 2-tailed; p =1.00) strain.

Only a very small fraction (2.9% of 69) of the *Lu. evansi* challenged with Nilo nose homogenates became infected (Table 7.2A).

Analysis of the pooled results from experiments 2 and 3 using the Nilo strain, clearly indicated dissimilarity between the patterns of infection rates and the intensity of infection of *Lu. longipalpis* and *Lu. evansi* at different time intervals (Figure 7.1). In the early post-feeding period, *Lu. evansi* and *Lu. longipalpis* exhibited their highest infection percentages (29.6% and 25.1%, respectively). However, infection intensity was higher in the *Lu. longipalpis* (2.5) than in the *Lu. evansi* (1.5) replicates. Subsequently, rates fell in both species (10.1% and 15.5%, respectively) but infection intensity increased (means of 2.3 and 3.4, respectively). After the fifth day post-feeding, both the infection rate and intensity dropped dramatically in *Lu. evansi* (2.9%; 1.3) while in *Lu. longipalpis* they were almost stable (14.5%; 3.5).

7.3.3 Parasite development

Despite the differences in infection percentages no clear differences were observed in the

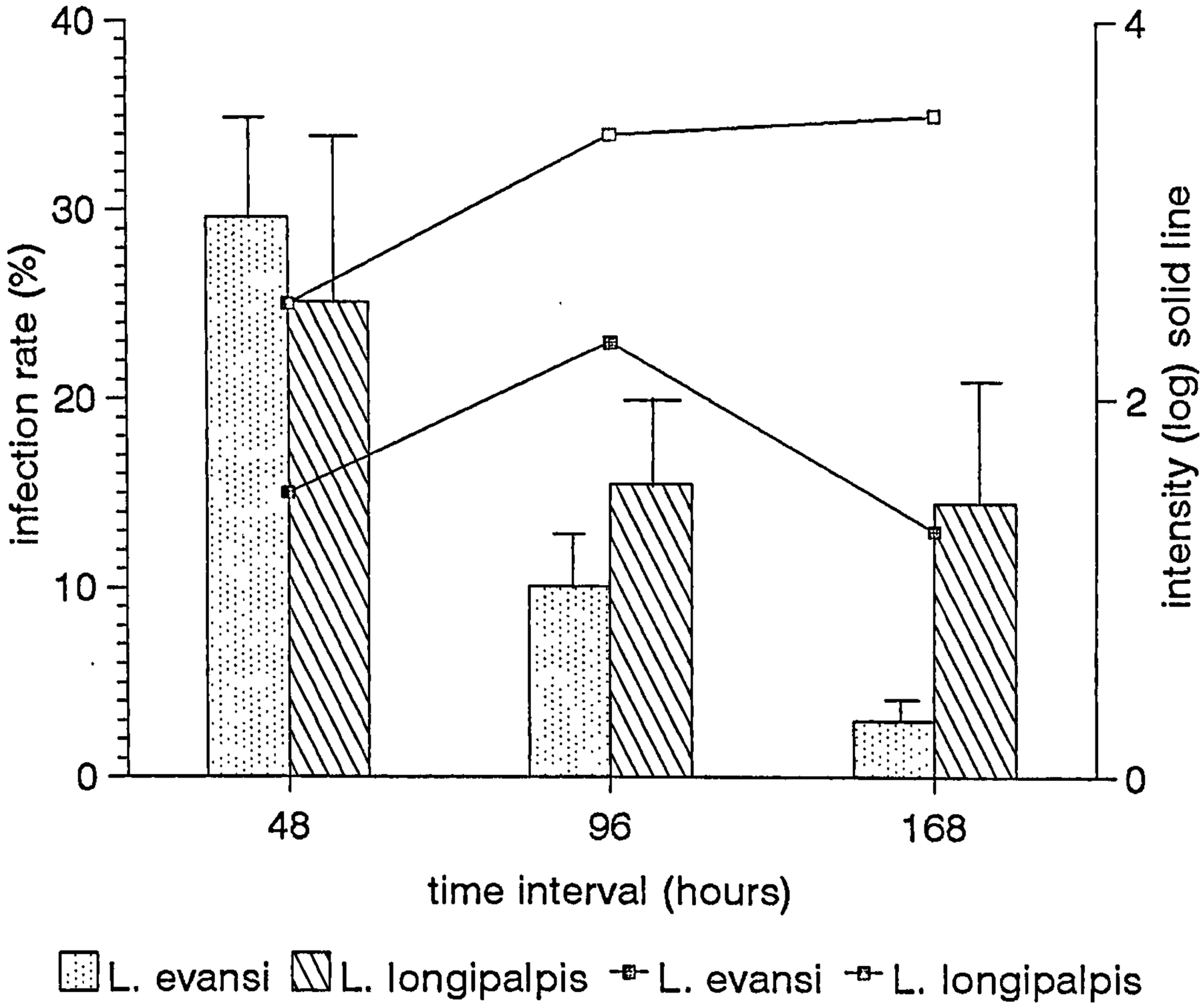


**Figure 7.1** Comparison of infection and intensity of infection between *Lu. evansi* and *Lu. longipalpis* from experimental infection experiment.

timing of transformation and division processes undergone by both promastigote and amastigote forms of Nilo strain in the guts of the two sandfly species. Hence, the following observations are based on the examination of infected flies with this strain, recording the differences only when they are worth noting.

At 12-24 h post-feeding, the blood meal including parasites (either promastigotes or amastigotes) were seen encapsulated in the peritrophic membrane. No conspicuous changes were observed in promastigote forms. Actually, it was not clear whether these divided or just underwent direct transformation to abundant "stumpy" forms in the blood-meal. Amastigote forms were very difficult to differentiate clearly because they formed clusters (nidi) within the undigested blood meal.

At the break-down of the peritrophic membrane, which occurred between the second and third day post-feeding, the parasites were released into the midgut but still they were embedded within the remnant blood. At this stage, an abundant mixture of slender, free nectomonad and almost rounded, non-motile paramastigote forms of variable size were seen throughout the

length of the posterior midgut. Some of them formed rosettes of up to four individuals, apparently in the process of division. Similarly, parasites with nectomonad characteristics were seen swimming freely in the hindgut of *Lu. evansi* but on no occasion, attached to any part of the pylorus or hindgut walls.

Between the fourth and fifth day after feeding, almost all flies had completed blood-meal digestion. This process was overall faster in *Lu. longipalpis* than in *Lu. evansi*. Different parasite forms were distributed along the alimentary canal; besides the previous described nectomonad and paramastigote forms, other nectomonads (slender but shorter) were recorded in the abdominal midgut lumen. Also, intermediate forms (pear-shaped) were seen forward in the anterior midgut. At this stage, a crucial event was worth noting: the parasite adhesion to the insect's anterior midgut wall. This was first noted in the infected *Lu. longipalpis*. Indeed, the pear-shaped forms appeared to orient their flagella towards the gut wall, suggesting some kind of attachment to this gut region. Conversely, in *Lu. evansi*, although adhesion was recorded, it was less obvious. Actually, it was estimated that in this fly, roughly 60% of parasites were not well attached but grouped in rosettes, forming light infections.

At the final stage (i.e. seventh-eighth day post-feeding), in both species clear, well defined but multiform haptomonads were attached to the thoracic midgut microvilli and the cuticular foregut region of *Lu. longipalpis*. In addition, in *Lu. longipalpis* very motile, short bodied, long flagellated forms (metacyclic?) were observed. They were swimming freely between the cardia and the stomodeal valve extension. In considerable contrast, infections in *Lu. evansi* were in the majority very light, with occasional haptomonad forms in the thoracic midgut attached to the gut wall. However, they appeared to be in division since small, very motile, probably "infective forms", were seen in the anterior midgut at the 8th day post feeding, though none of them were seen passing the stomodeal valve.

### 6.3.4 Transmission assays

A total of 25 flies of both species took a second blood-meal on hamsters (Table 7.3). The *Lu. longipalpis* re-feeding rate was higher (76%) than *Lu. evansi* (24%). Dissections revealed no parasites in the refed *Lu. evansi* while 31.6% of *Lu. longipalpis* were found harbouring promastigotes. However, not one of the three bitten hamsters showed signs of infection.

A total 38 flies of both species were used in the forced-feeding procedure (Table 7.3). This was effective in recovering "infective" and paramastigote forms from four *Lu. longipalpis*

and just one *Lu. evansi*.

**Table 7.3** Pooled results of experimental transmission (refeeding on healthy hamsters) and forced feeding.

| | *Lutzomyia evansi* | | *Lutzomyia longipalpis* | |
|---|---|---|---|---|
| | N | +ve | N | +ve |
| Refed on hamsters | 6 | 0 | 19 | 6 |
| Forced feeding | 18 | 1 | 20 | 4 |
| TOTAL | 24 | 1 | 39 | 10 |

7.3.5 Analysis of survival and egg development

Although mortality was almost nil in flies fed directly on infected hamsters (experiment 1) it was higher in *Lu. evansi* fed on L-12 hamsters ($\chi^2$= 4.31; p= 0.03) than in *Lu. longipalpis*. Also, the group of *Lu. evansi* flies fed with Nilo promastigotes (experiment 2) presented a significantly higher mortality compared with its pairs *Lu. longipalpis* (Fisher 1-tailed= 0.0022). Conversely, no differences in mortality were found using promastigotes of the L-12 strain. On the other hand, despite the very light infections exhibited by *Lu. evansi* and *Lu. longipalpis* fed on spleen homogenate of both parasite strains (experiment 3), a high mortality rate was noted in both sandfly species at the fifth day post feeding. Mortality rates were higher in *Lu. evansi* than in *Lu. longipalpis* ($\chi^2$= 43.37 to L-12 and 8.91 to Nilo; $p < 0.05$). Finally, in the experiments using the Nilo nose homogenate, it was found that mortality rates were equal in infected (n= 69) and uninfected (n= 60) *Lu. evansi*. In summary, disregarding the parasite strain employed to infect flies, overall mortality rates were higher in the infected *Lu. evansi* than in *Lu. longipalpis*.

Uninfected female *Lu. evansi* (n= 65) fed on hamsters developed in average of 27.9 +/- 19.4 (range= 5-56) eggs. In comparison, two batches of 10 and 9 infected flies (from experiments 2 and 3) developed an average of 16.9 +/- 10.6 (range= 0-30) and 19.2 +/- 6.5 (range= 12-30) eggs, respectively.

## 7.4 Discussion

The results suggest that at least one *Sauroleishmania* and two *Leishmania* species are circulating among sandfly populations in El Contento area. Unfortunately the parasites could not be characterized. Nevertheless, from the parasites location in the sandfly gut it is possible to gather some information. For instance, the hypopylarian parasites found in the gut of several specimens of *Lu. cayennensis* strongly suggests we were dealing with a *Sauroleishmania* species. This finding is strengthened by the fact that specimens from *Lu. cayennensis* group have been found infected with *Sauroleishmania* in nature (Lewis, 1975) and the continual reports of this species as a reptile feeder (Christensen & Herrer, 1983; Young & Duncan, 1994). However, we have to point out that in Colombia and Venezuela, *Lu. cayennensis* has been found harbouring trypanosomes (Ryan et al., 1987; Deane et al. 1978) and that in our host preference studies we found this species feeding on human and bovines (see Chapter 4). Despite the biological importance of the *Lu. cayennensis* infections, only those infections recorded in *Lu. evansi* have particular importance in the AVL dynamics, henceforth discussion will focus on these.

The anterior position of the parasites found in two naturally infected *Lu. evansi* resembles the development of a typical suprapylarian *Leishmania* (probably *L. chagasi*, the main strain circulating in the area) while that from the third fly corresponds to a peripylarian species (possibly *L. panamensis*).

The low proportion of infected flies detected during our study was expected taking into account that, with exception of some areas in Brazil, sandfly infection rates with *L. chagasi* are reported to be very low and occur cyclically (Sherlock & Miranda, 1993). Therefore, not surprisingly, in few cases has the natural vector/parasite association been directly established. Actually, our results agree well with preliminary work in El Contento by Travi et al. (in press) who, with difficulties, achieved isolation and identification of *L. chagasi* from a few wild-caught *Lu. evansi*. In their work, from a total of 4,116 *Lu. evansi* dissected, six specimens (0.14%) were found naturally infected with promastigote forms, three of them being characterized as *L. chagasi*. In their turn, the low infection rate reported in the El Contento area is consistent with that recorded in sandfly populations transmitting both visceral (Morrison, 1994) and cutaneous leishmaniasis elsewhere in Colombia (Travi et al ., 1988). From all of these findings, it emerges that in El Contento, the study of the natural sandfly infection rates deserves further evaluation but this would be aided with modern DNA probes, such as the Lmet-2 probe, specific for the *L. donovani* complex (Howard et al., 1991) or the powerful PCR methods (Dr

D. Barker, pers. comm.).

Results from the experimental infections represent the first evaluation and comparison of the life cycle of *L. chagasi* in its alternate vector, *Lu. evansi*. It was shown that under experimental conditions both *Lu. longipalpis* and *Lu. evansi* support suprapylarian development of *L. chagasi* initiated either by spleen homogenates or promastigote cultures. Also, in both cases, the parasite infection appeared to have detrimental effects on the survival of the infected flies. The latest results are consistent with the collected experimental evidence suggesting that *Leishmania* (as well on other Trypanosomatidae) can be pathogenic to their vectors (D'Alessandro, 1976; Molyneux, 1983; Killick-Kendrick, 1985; El Sawaf et al., 1994). The last authors found that infection with promastigotes of *L. major* and *L. infantum* caused a decrease in longevity and egg production on *P. papatasi* and *P. langeroni*. However, no one has established the mechanism for this detrimental effect (El Sawaf et al ., 1994).

A multifactorial effect might have precluded the normal development of *L. chagasi* in *Lu. evansi*. During their first stage of development, nearly all infections showed an increase in infected individuals followed by some decrease around the fifth day, following excretion of the blood-meal. This represents an important loss of parasites, which might be explained in terms of either activity of some gut enzymes (Dillon & Lane, 1993) or to an inefficient adhesion of parasite forms to the midgut walls. These events might not be mutually exclusive but they might act sequentially. In fact, observation of flies containing only dead parasites or aberrant promastigote-like forms "trapped" within undigested blood, suggests the existence of an enzymatic barrier which might partially account for the irregular development and low infectivity rates observed with the L-12 strain in both sandfly species. Borovsky & Schlein (1987), showed that a peak of protease activity, at 30h post feeding in *P. papatasi* with *L. donovani*, inhibited the growth of this parasite in the sandfly.

Two other events indicate that the low infectivity in *Lu. evansi* might be explained in terms of the parasite's inability to survive in the insect gut. Firstly, the observation of clusters of parasites within the blood-meal suggests that parasites might strategically "employ" the formation of large masses to avoid excretion with the digested blood. This corroborates observations by Walters et al. (1987), who observed a similar phenomenon in the *Le. mexicana/Lu.abonnenci* association. Secondly, the observation of some unattached

promastigotes in the hindgut region, apparently unable to establish in this area, and which were eventually expelled with the faeces. Since this phenomenon did not occur in *Lu. longipalpis*, even in heavy infections, and because *Lu. evansi* infections were light, we exclude the possibility of parasite "overflow" as reported in previous works (Walters et al., 1987; Williams, 1970). Undoubtedly, the capacity of the parasites to bind to specific areas of the gut is a prime requisite for any successful parasite/host association. As pointed out by Molyneux (1983), it is unlikely that a stable number of parasites will be found in the insect's lumen without attachment since the rapid movement of gut contents would expel any unattached parasites. Recently, much evidence has shown the important role of lipophosphoglycan (LPG), the most abundant promastigote surface molecule, in the specific attachment and detachment of *L. major* in the insect's midgut epithelial cells (Davies et al ., 1990; Pimenta et al., 1994; Saraiva et al., 1995).

In our case, since (1) the *L. chagasi* infections never flourished as well in *Lu. evansi* as they did in *Lu. longipalpis*, (2) the mortality rate in infected flies was overall greater in *Lu. evansi* than in *Lu. longipalpis*, and (3) infected *Lu. evansi* matured significantly lower numbers of eggs compared with uninfected flies of the same species, the evidence indicates that *L. chagasi* is better adapted to *Lu. longipalpis* than *Lu. evansi*. Assuming that the historical duration of an association is equivalent to co-adaptation (ie. even a large number of parasites will not produce significant pathological effect on its specific vector), our results suggest that *L. evansi* is a more recent vector of this parasite than *L. longipalpis*. Price (1980) defines the effective environment of a parasite as the patch where it is currently situated and another patch that the parasite or its progeny must reach in order to find new hosts. In metaxenic parasites (like *Leishmania*) this prime goal is reached if they are able to undergo and successfully adapt to developmental changes through both, the invertebrate and vertebrate hosts. If so, with time, it would led to a high degree of co-evolutionary radiation of the parasite in a greater number of vectors. Excellent examples of this event are found in the wide radiation reached by *L. infantum* and *L. braziliensis*, parasites respectively natives from the Old and New World (Table 7.4). Conversely, since its possible recent introduction into the Americas, *L. chagasi*, the causative agent of AVL, has poorly radiated in the local sandfly fauna, at present *Lu. longipalpis* and *L. evansi* are its only known vectors. Thus, as pointed by Price (1980) parasites can be extremely useful in unravelling the phylogenetic relationships of their hosts.

Apart from the wider conceptual issues, our data have shown that *L. chagasi* parasites

**Table 7.4** Natural vectors of *Leishmania infantum* and *L. braziliensis.*

| Old World *Leishmania infantum* | New World *Leishmania braziliensis* |
|---|---|
| *Phlebotomus (Adlerius) chinensis* | *Lutzomyia (Nyssomyia) intermedia* |
| *P. (A.) longiductus* | *Lu. (N.) whitmanni* |
| *P. (Larroussius) ariasi* | *Lu. (Pintomyia) pessoai* |
| *P. (L.) kandelakii* | *Lu. (Psychodopygus) amazonensis* |
| *P. (L.) langeroni* | *Lu. (P.) ayrozai* |
| *P. (L.) longicuspis* | *Lu. (P.) carrerai* |
| *P. (L.) neglectus* | *Lu. (P.) complexus* |
| *P. (L.) perfiliewi* | *Lu. (P.) llanosmartinsi* |
| *P. (L.) perniciosus* | *Lu. (P.) panamensis* |
| *P. (L.) smirnovi* | *Lu. (P.) paraensis* |
| *P. (L.) tobbi* | *Lu. (P.) wellcomei* |
| *P. (L.) transcaucasicus* | *Lu. (P.) yucumensis* |
| | *Lu. migonei* |
| | *Lu. spinicrassa* |

bind to the gut walls of *Lu. evansi* after blood meal digestion, then detach and finally, migrate towards the foregut parts. Clearly, additional and specialized studies are required to establish the actual nature of the LPG modulated binding during the development of the *L. chagasi* within this "permissive" vector species. In summary, taking into account the evidence that *Lu. evansi* is a poor host of *L. chagasi*, our field and experimental findings strongly support the hypothesis

that AVL in the Caribbean coast of Colombia (and probably in some areas of Venezuela) is transmitted by *Lu. evansi*. This sandfly fulfils some of the basic criteria enumerated by Killick-Kendrick & Ward (1981) to incriminate any sandfly as vector of human leishmaniasis. In the first instance, the *Lu. evansi* population is sufficiently abundant and active to maintain sylvatic and domiciliary AVL transmission. Secondly, in the SAS area, this sandfly is anthropophilic and is the main intradomiciliary human-biter. Thirdly, the experimental data shows *L. chagasi* develops in *Lu. evansi* (ie. parasite transformation, escape from peritrophic membrane and nectomonad/haptomonad colonization of midgut and foregut areas) yielding viable metacyclic forms. Although no direct evidence of the presence of infective forms in the cibarium and pharynx was shown, the finding of promastigotes during forced feeding experiments suggests that the parasites are disgorged during feeding. Finally and most important of all the occurrence of *Lu. evansi* naturally infected is sufficient evidence of transmission in the Caribbean coast area.

# CHAPTER 8

# BIOSYSTEMATICS

## 8.1 Background

The *Lutzomyia verrucarum* group includes a total of 28 species (Young & Duncan, 1994). Females are characterized by striated, saclike paired spermathecae while male genitalia exhibit a basal tuft on the coxite and a simple paramere. Taxonomically, the *verrucarum* group was divided by Theodor (1965) into the *serrana* and *verrucarum* series. The latter includes many species whose females are isomorphic, indistinguishable using morphological criteria (Feliciangeli et al., 1992).

The involvement of *Lutzomyia evansi*, a species belonging to the *verrucarum* group, in the transmission of visceral leishmaniasis in Colombia (Travi et al., 1990) has drawn our attention to the possible vectorial role of this species elsewhere. Originally *Lu. evansi* was described by Núñez-Tovar in 1923, from Mariara, a small village in Carabobo State, in Venezuela (Carbonell, 1938). In Venezuela, *Lu. evansi* is widespread, colonizing almost all types of ecological zones, except humid forest (Feliciangeli et al., 1992). In Colombia, its distribution is more restricted to semi-arid zones in the north of the country (Young & Duncan, 1994; Young, 1979). The species has also been reported in almost all Central American countries, though there is no evidence of its presence in Panama despite intensive sandfly studies there. The known disjunct distribution of *Lu. evansi* is shown in Figure 8.1

To determine whether *Lu. evansi* is a homogeneous species (ie. whether knowledge of the biology of Colombian *Lu. evansi* populations can be applied to populations of this species throughout its geographical range ) the following study was initiated to compare Central American and South American populations of *Lu. evansi*. Comparisons were made at two levels: morphologically, using conventional identification keys (Young & Duncan,

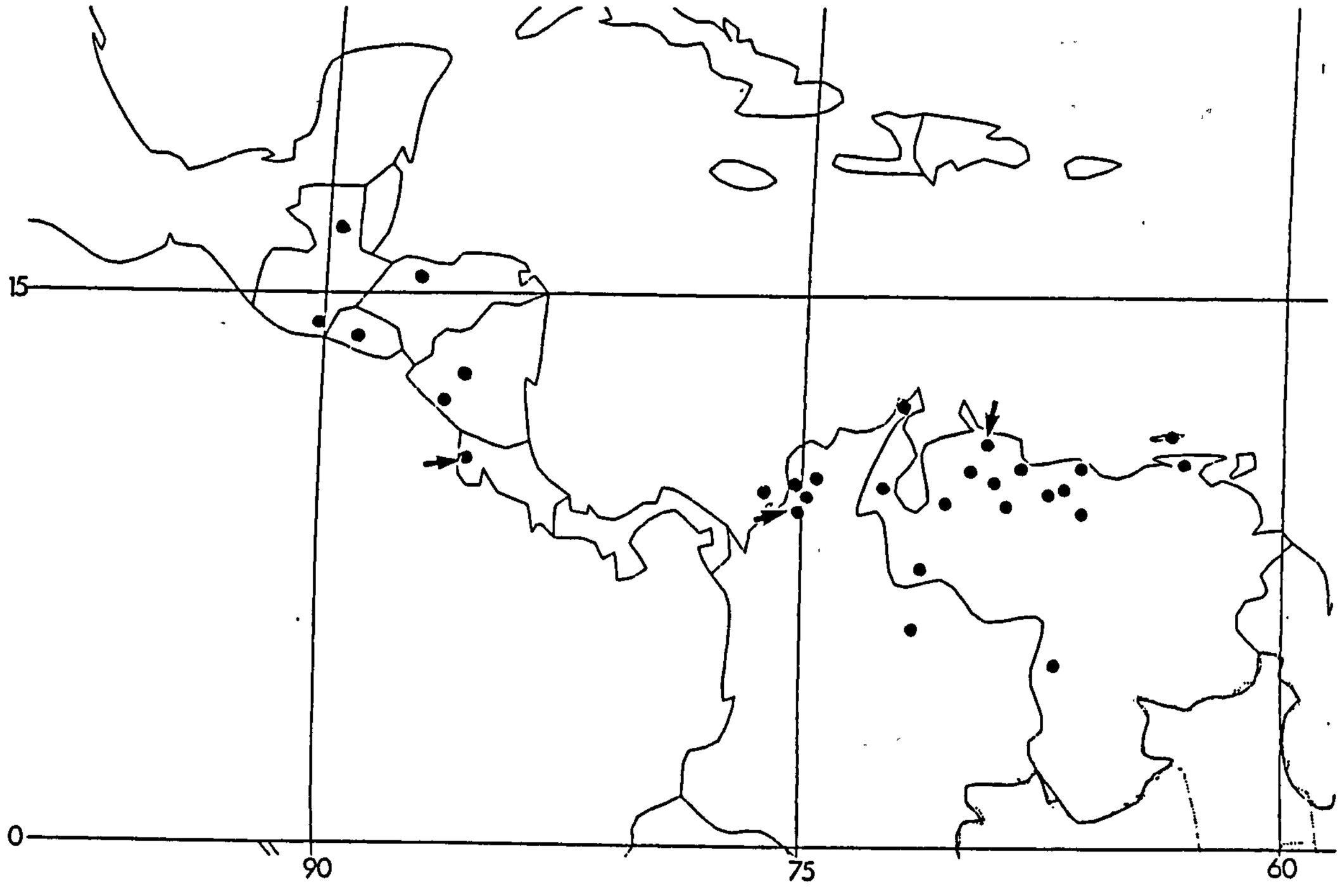


**Figure 8.1** Known geographical distribution of *Lu. evansi* (dots). Arrows indicate places where samples were taken for the present study.

1994) and genetically by using isozymatic (Kreutzer et al. 1990) and mitochondrial (Ready et al., submitted) criteria.

## 8.2 Methods

### 8.2.1 Sites and sandfly collections:

Colombian *Lu. evansi* were captured exclusively by Shannon trap in three villages at San Andrés de Sotavento (9°09'N, 75°31'W) through 1993-94: El Contento (several collections)- Gardenias (2 collections) and Vidales (1 collection). Sandfly captures were also made in June 1993 in Brasilito, Guanacaste Province, Costa Rica (10°37'N, 85°26'W) and September 1993 in Vereda Guayabita, Carabobo State, Venezuela (10° 26'N, 68°01'W). Alcohol-preserved specimens from Ticuantepé (12°01'N, 86°12'W), a locality near Managua, Nicaragua, kindly provided by Dr. F. Collantes (Universidad de Murcia, Spain) were also

included but only for morphological and mitochondrial analysis. Hence, during the present study, both contiguous and allopatric populations were analysed. The geographical locations of collection sites are shown in Figure 8.1. Caught specimens were anaesthetized with smoke and immediately frozen in liquid nitrogen. In all cases random sub-samples were taken as vouchers.

### 8.2.2 Morphological comparison

Voucher specimens from Venezuelan, Colombian and Costa Rican collections (20 from each catch) were cleared in lactophenol solution and wherever possible mounted using Berlese medium. Since the Costa Rican samples contained few *Lu. evansi,* batches of flies were placed on a Saran wrap paper-lined petri dish (kept cool on ice) and rapidly sorted by external morphological characters. Terminal abdominal segments containing spermathecae were removed and cleared from those specimens exhibiting features of *Lu. verrucarum* group flies. The rest of the body was preserved for PCR analysis. Individuals from Nicaragua were rinsed in distilled water, then transferred and mounted with Berlese medium. Species identification was assessed by Young & Duncan's key for *verrucarum* species (Young & Duncan, 1994; p. 171-175) using a light microscope (Stemi SV6 Zeiss®) attached to a Video-plan unit (Kontron Elektronik®).

### 8.2.3 Genetic comparison

To test the distinctness of *Lu. evansi* populations , both isozymatic and mitochondrial DNA (mtDNA) patterns were compared between Colombian, Venezuelan and Costa Rican sandfly samples. Therefore, there were two scales of the study: analysis of variation of three Colombian populations and variation between *Lu. evansi* from Venezuela and Costa Rica and Colombia.

8.2.3.1 Isozyme technique: Batches of 20 unfed individuals (females or males) were stored and shipped in liquid nitrogen (-196°C) or dry ice (-69°C) before final storage at −70°C until used for electrophoresis. A total of 22 enzymatic loci were assayed based on previous work by Kreutzer **et al.** (1990); Petersen (1982; 1984); Miles & Ward (1978) and Ward **et al.** (1981). The Enzyme Commission (E.C.) numbers, names and abbreviations for these enzymes together with a list of the grinding and running buffers tested are given in Tables 2.1. All of them were prepared beforehand and kept refrigerated. The Super Z Helena® (Beaumont,

Texas) electrophoresis kit was used. The outer compartments of the chambers were filled with 100ml of electrode buffer, stored in the refrigerator and changed when reduction in resolution was observed.

* Preparation of the samples: isozymes procedures were based on the recipes described in Helena's handbook (Hebert & Beaton,1989), adding slight modifications from Kreutzer et al. (1990) and Petersen (1982; 1984). Briefly, single flies were placed in clean labelled 1-1.5ml Eppendorf tubes and kept on dry ice. Each fly was ground in 15µl of cooled 1x of DTT grinding solution (ie. 2mM dithiotreitol, 2mM amino-n-caproic acid, 2 mM EDTA)[1] using a disposable Gilson tip per sample. Immediately, 5µl of the homogenate was transferred to one of the wells of the sample well-plate, held on ice. Sample remains were sorted frozen at -70°C until further use.

* Preparation and loading of plates: Cellulose acetate (Opthipor® plates 12 or 8 wells) was used as supporting medium. Each plate was labelled indicating the date, name of the enzyme analysed and cathode end. Carefully, plates were immersed in a 500ml beaker containing electrode buffer and separated with glass rods. Soaking was conducted at least for 20min. When loading, each plate was withdrawn and blotted dry using 3M® chromatography paper. Rapidly, the acetate was placed shiny-side down and cathodically aligned on the aligning base. Then, the applicator was loaded with the samples and applied immediately to the plate by depressing the tips (number of applications ranged between 1 and 3). The plate was placed acetate side down on the wick-supporting bridges of the electrophoresis chamber. Two frozen sticks were placed in the inner compartments before running.

* Electrophoresis, staining and storage: Time and voltage conditions for each electrophoresis were 15-20min at 200 volts (100 volts in esterase), respectively. During the running time, the chemicals for the appropriate stains were mixed with the exception of the stain activator. The plate was placed on a piece of glass, the enzyme activator and 2ml of agar solution were added to the stain mix, mixed and poured over the plate. After one minute, the plate was placed in an incubator at 37°C. Finally, after a few minutes, plates were washed in distilled $H_2O$ for 24 hours, dried and stored in plastic bags in the dark. A homogenate of a clone of *Myzus persicae* (Aphididae) or *Ph. papatasi* was used as standard control.

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[1] Although the grinding solutions employed by Kreutzer et al. (1990) and Petersen (1982) were tested, best results were obtained using either DTT or TG (Tris-Glycine).

**Table 8.1** Abbreviation, EC code and reagents for each of the enzyme systems assayed with *Lu. evansi* from Colombia and Venezuela. Based on the recipes described in Helena's handbook (Hebert & Beaton,1989). Buffers: Tris Glycine (TG pH = 8.5) 30g Trizma; 144 g Glycine in 1L $dH_2O$. Tris-Maleate TM pH= 7.8): 6.06g Trizma; 2.32g Maleic acid in 1L $dH_2O$. Solution 1: 200ml 0.1 M phosphate pH=7.0; 10mg Pyridoxal -5-Phosphate; 460mg L-Aspartic acid; 260mg α-ketoglutaric acid pH=7.4. Malic substrate (pH= 8.0): 180mL $dH_2O$; 20mg Trizma HCL pH=9.0; 3.68 g L-malic acid. d= 1 drop.

| | ADH | AO | EST | GPDH | GOT | 6GPDH | HEX | IDH | MDH | ME | MPI | PGI | PGM | PEP | TRE | XDH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EC Number | 1.1.1.1.1. | 1.2.3.1 | 3..1.1.1 | 1.1.1.8 | 2.6.1.1 | 1.1.1.44 | 2.7.1.1 | 1.1.1.42 | 1.1.1.37 | 1.1.1.40 | 5.3.1.8 | 5.3.1.9 | 2.7.5.1 | 3.4.1.1 | 3.2.1.28 | 1.2.1.37 |
| BUFFER | TG | TM | TM | TM | TG | TM | TM | TM | TM | TM | TM | TG | TG | TG | TM | TM |
| Tris HCL pH8.0 | | 0.6ml | | 0.6ml | | 0.6ml | | | 1.0ml | 0.6ml | 1.0ml | 1.0ml | 1.0ml | | 1.0ml | 1.0ml |
| Tris HCL ph7.0 | 0.6ml | | | | | | 0.6ml | 1.0ml | | | | | | | | |
| 0.1 Tris maleate5.3 | | | 2ml | | | | | | | | | | | | | |
| Solution 1* | | | | | 3ml | | | | | | | | | | | |
| 0.02M $Na_2HPO_4$ | | | | | | | | | | | | | | 2.0ml | | |
| NAD | 1.5ml | 1.5ml | | 1.5ml | | | 1.5ml | | 1.5ml | | | | | | | |
| NADP | | | | | | 1.5ml | | 1.5ml | | 1.5ml | 1.5ml | 1.5ml | 1.5ml | | 1.5ml | 1.5ml |
| etOH | 3d | | | | | | | | | | | | | | | |
| Benzaldehyde | | 1d | | | | | | | | | | | | | | |
| αnapthyl acetate or 4methylumbellifelyl acetate in acetone | | | 200µl | | | | | | | | | | | | | |
| Fast Blue BB salt (Saturated Solution) | | | | | 10d | | | | | | | | | | | |
| Fast Red(Saturated) | | | 10d | | | | | | | | | | | | | |
| Trehalose | | | | | | | | | | | | | | | 5d | |
| αGlycerolphosph. | | | | 22d | | | | | | | | | | | | |

| | ADH | AO | EST | GPDH | GOT | 6GPDH | HEX | IDH | MDH | ME | MPI | PGI | PGM | PEP | TRE | XDH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ATP+DGlucose Sol | | | | | | | 16d | | | | | | | | | |
| ATP | | | | | | | | | | | | | | | 4d | |
| DLIsocitric acid | | | | | | | | 15d | | | | | | | | |
| Malic substrate | | | | | | | | | 13d | 12d | | | | | | |
| DMannse6Phospha. | | | | | | | | | | | 5d | | | | | |
| o-Dianisidine | | | | | | | | | | | | | | 8d | | |
| Glucose1Phosphate | | | | | | | | | | | | | 5d | | | |
| 6Phosphogluconic a | | | | | | 6d | | | | | | 5d | | | | |
| Fructose6Phosphate | | | | | | | | | | | 5d | | | | | |
| $MgCl_2$ | | | | | | 6d | 5d | 8d | | 2d | | | 5d | 2d | 5d | |
| MTT | 5d | 5d | | 5d | | 5d | 5d | 5d | 5d | 5d | 5d | 5d | 5d | | 5d | 5d |
| PMS | 5d | 5d | | 5d | | 5d | 5d | 5d | 5d | 5d | 5d | 5d | 5d | | 5d | 5d |
| G6PDH | | | | | | | 10µl | | | | 20µl | 5µl | 20µl | | 5µl | |
| PGI | | | | | | | | | | | 5µl | | | | | |
| Hexokinase | | | | | | | | | | | | | | | 5µl | |
| Hypoxanthine | | | | | | | | | | | | | | | | 20d |
| Leu-Lys or Leu-Ala dipeptide | | | | | | | | | | | | | | 8d | | |
| Lamino acid oxid. | | | | | | | | | | | | | | 8d | | |
| Peroxidase | | | | | | | | | | | | | | 4d | | |

8.2.3.2 PCR technique

Approximately 550 base pairs (bp) of the mitochondrial genome was amplified using a forward primer (a 20-mer starting at position 11214 of *Anopheles gambiae* mtDNA (Beard et al., 1993)) annealing to the cytochrome B gene and a reverse primer binding to the NAD1 gene (a 26-mer starting at position 11759 of *Anopheles gambiae* mtDNA (Beard et al., 1993).

* DNA extraction: To break down and lyse tissues, 1-1.5ml Eppendorf vials with frozen individuals (-70°C) were allowed to warm to room temperature (18°C) for 1min and then ground in a mix of 1x grinding buffer (0.1M Tris-HCl pH7.5; 0.6M NaCl and 0.1M EDTA), 0.15mM spermine/0.15mM spermidine, 5% (w/v) sucrose in $dH_2O$. To denature the proteins associated with the DNA, 10µl of SDS mix (0.8M Tris-HCl pH9.0 + 0.27µl EDTA) was immediately added to each vial, followed by tapping and a short spin (14Krpm), then incubation in a waterbath (65°C) for an hour. Afterwards, samples were cooled on ice, spun, mixed with 30µl of an ice-cold solution of 8M potassium acetate, left for 2h, followed by a 2min spin before the DNA containing supernatant was transferred to a new labelled vial, leaving behind SDS-protein complexes. DNA was precipitated overnight in 400µl of 95% ethanol (EtOH) at −20°C. The following day, tubes were vortexed and warmed to room temperature. The precipitated DNA was washed three times in 75% EtOH, each wash consisting of vortexing, 5min spin at 14Krpm and blotting on paper towel. The samples were dried under vacuum for 5min. The pellet was totally redissolved in 15µl $dH_2O$ for 10-15min.

* Electrophoresis: To assess the presence of DNA, samples were electrophoresed at constant voltage (50/70v) for 2h, in a 0.8% micro-agarose gel in TBE buffer. DNA bands were visualised on a UV trans-illuminator.

* PCR: Positive DNA samples were amplified using TaqDNA polymerase. 2µl of a DNA sample was added to 47µl of reaction mix (100µl Promega Thermophilic Buffer; 60µl Promega Magnesium Chloride (25mM); 6.0µl each of dATP (10mM), dCTP, dGTP and dTTP; 20µl Forward primer (0.5µg/µl); 20µl Reverse primer (0.5µg/µl) and 716µl sterile double $dH_2O$ were added for a '1000µl' master mix with Promega TaqDNA. Thus the final volume was 940µl). Each reaction was overlaid with 50µl heavy mineral oil and the DNA denaturated by heating the samples at 94°C for 3min in a thermocycling block (Omnigene, Hybaid), with a hold at 80°C. Immediately 1µl of Taq-polymerase (1 unit/1µl) was added to each tube and replaced in the block. The thermocycling was restarted with the following program: 5 cycles: 94°C 30s; 40°C 30s; 72°C 2min and 35 cycles: 94°C 30s; 44°C 30s; 72°C 2min making a total of 40 cycles. After PCR, the oil was extracted using chloroform. Then,

the PCR products were mixed with TBE loading buffer, electrophoresed in a 1.2% agarose gel (at 60v for 3h) and finally visualised on an UV transilluminator. The products were recorded using a Polaroid film (No 665 with orange filter).

* Cleaning: DNA bands were cleaned up using the GeneClean® kit (Bio101 Inc), following the instructions and recommendations of the manufacturer. Briefly, DNA slices from the gel were placed into a pre-weighted vial. The volume of each gel slice was estimated by subtracting the vial weight from the gel + vial weight. Sodium iodine solution was added to each sample and placed at 45°C in a waterbath for 5min. The content of each vial was mixed and returned to the bath; this procedure was repeated once. A 5µl glassmilk suspension was added to each solution, mixed and left on ice for 5min with periodical mixes. To recover the DNA, the glassmilk/DNA mix was diluted in $dH_2O$, left in a waterbath at 50°C and spun. The purified PCR product was finally sequenced.

* Sequencing: The ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction kit was used following the instructions of the manufactures (Perkin Elmer). Basically, 20µl of PCR product was precipitated in an EtOH solution of sodium acetate (2.0 µl 3M sodium acetate pH 4.6 + 50µl 95% EtOH). After a short vortex, each sample was placed on ice for 10min and then centrifuged at maximum speed (14 Krpm). The EtOH was tipped away and the pellet rinsed with 70% EtOH, which was tipped away after centrifugation and finally dried in a vacuum. The products were loaded and sequenced in a ABI 373A semi-automated sequencing apparatus. Sequence data were read and aligned using the Sequence Editor (SeqEd) and Navigator Software. Phylogenetic reconstruction and comparisons were done using PAUP (Phylogenetic Analysis Using Parsimony) Version 3.1 (Swofford, 1991) using the homologous sequence of *Lu. whitmani* as an out-group.

## 8.3 Results

### 8.3.1 Morphological comparison

A total of 40 individuals (20♂♂ and 20♀♀) from each of the populations of Colombia and Venezuela, 11 ( 7♂♂ and 4♀♀) from Nicaragua and 3♀♀ (spermathecae only) from Costa Rica, were used in this analysis. Overall, although size variations were observed among specimens such variation did not modify the morphological characters examined using the dichotomous key to suggest presence of distinct populations.

8.3.2 Isozyme comparison

Only Colombian and Venezuelan populations were analyzed. From the 18 isozymatic systems tested, $EST_1$ (using o-napthyl acetate as substrate) showed the highest polymorphism but with uninterpretable bands. Activity but unclear patterns, sometimes difficult to read and assess was recorded in PEP (L-leucyl-L-alanine hydrate) ADH, AO, ME, MPI, XDH and TRE. No visible bands or absence of enzymatic activity was observed in PEP (Lysine-Leucine hydrate), GOT, HEX and $EST_2$. Finally, the enzymes PGM, PGI, IDH, MDH, GPDH and 6GPDH showed band patterns consistent with two or more isozymes of easy interpretation. The number of flies and their respective allele and genotype frequencies for each population is given in Table 8.2. In MDH two forms, mitochondrial and cytoplasmic (fastest) were revealed but only the second was scored. A contingency chi-square analysis of allele frequencies within Colombian populations (ie. Gardenias and El Contento) showed no significant differences from one to another in all loci studied ($p>0.05$). Analysis to determine genotype balance in both Colombia and Venezuela populations revealed that with the exception of PGM, GPDH and 6GPDH, whose frequencies distorted from Hardy-Weinberg equilibrium ($p< 0.05$), the remaining loci did not depart from the expectations of panmixis. Further analysis showed that some of the expected genotype frequencies in both enzyme systems were less than 5 (Table 8.3). Therefore, data sets of both Colombia and Venezuela populations were combined to guarantee meaningful comparisons with $\chi^2$ tests.

Comparisons of the observed and expected frequencies showed no significant differences for GPDH when samples were combined ($\chi^2=0.86$; df=1; $p= 0.35$). In contrast, an almost significant variation was detected ($\chi^2= 4.74$; df= 2; $p= 0.09$) for 6GPDH (Table 8.4).

8.3.3 Mitochondrial comparison

With the exception of the Nicaraguan *Lu. evansi* population (alcohol preserved flies), DNA was successfully extracted from the remaining three populations (Figure 8.2). Clustal alignment of 384bp fragments in *Lu. evansi* populations from Colombia (E195Col), Venezuela (E194Ven) (each based on 2 sequenced PCR products from 2 sandflies) and Costa Rica (E359CR1J) (based on 1 PCR product only), showed a total of 34bp varied among the three populations (Figure 8.3). From these, the majority (97%) were located in the cytochrome B

**Table 8.2** Allele and genotype frequencies of six enzyme systems in *Lu. evansi* populations from Colombia and Venezuela.

| | Allelic frequencies | | Observed (Expected) frequencies genotypes | | |
|---|---|---|---|---|---|
| **Country** | **Colombia** | **Venezuela** | **Genotype** | **Colombia** | **Venezuela** |
| PGM | 10 | 6 | | | |
| a | 0.050 | 0 | aa | 0 (0) | |
| | | | ab | 1 (0.368) | |
| | | | ac | 0 (0.579) | |
| | | | ad | 0 (0.053) | |
| b | 0.350 | 0.167 | bb | 3 (1.105) | 1 (0.091) |
| | | | bc | 0 (4.053) | 0 (1.636) |
| | | | bd | 0 (0.368) | |
| | | | be | | 0 (0.182) |
| c | 0.550 | 0.750 | cc | 5 (2.895) | 4 (3.273) |
| | | | cd | 1 (0.597) | |
| | | | ce | | 1 (0.818) |
| d | 0.050 | 0 | dd | 0 (0) | |
| e | 0 | 0.083 | ee | | 0 (0) |
| PGI | 25 | 25 | | | |
| a | 0.060 | 0.1 | aa | 0 (0.061) | 1 (0.204) |
| | | | ab | 3 (2.755) | 3 (4.184) |
| | | | ac | 0 (0.122) | 0 (0.306) |
| | | | ad | | 0 (0.102) |
| b | 0.90 | 0.820 | bb | 20 (20.204) | 17 (16.735) |
| | | | bc | 2 (1.837) | 3 (2.510) |
| | | | bd | | 1 (0.837) |
| c | 0.040 | 0.060 | cc | 0 (0.020) | 0 (0.610) |
| | | | cd | | 0 (0.610) |
| d | 0 | 0.020 | dd | | 0 (0) |

Continued

**Table 8.2 (contd)** Frequencies of alleles and genotypes of six enzyme systems in *Lu. evansi* populations from Colombia and Venezuela.

| | Allelic frequencies | | Observed (Expected) frequencies genotypes | | |
|---|---|---|---|---|---|
| **Country** | **Colombia** | **Venezuela** | **Genotype** | **Colombia** | **Venezuela** |
| GPDH | 32 | 32 | | | |
| a | 0.234 | 0.234 | aa | 1 (1.667) | 6 (1.667) |
| | | | ab | 13 (11.667) | 3 (11.667) |
| b | 0.766 | 0.766 | bb | 18 (18.667) | 23 (18.667) |
| IDH | 36 | 18 | | | |
| a | 0.111 | 0.389 | aa | 1 (0.394) | 4 (2.60) |
| | | | ab | 6 (7.211) | 6 (8.80) |
| b | 0.889 | 0.611 | bb | 29 (28.394) | 8 (6.60) |
| 6GPDH | 16 | 13 | | | |
| a | 0.375 | 0.731 | aa | 5 (2.129) | 9 (6.840) |
| | | | ab | 2 (6.194) | 1 (5.320) |
| | | | ac | 0 (1.548) | |
| b | 0.50 | 0.269 | bb | 7 (3.871) | 3 (0.840) |
| | | | bc | 0 (2.065) | |
| c | 0.125 | 0.0 | cc | 2 (0.194) | |
| MDH | 27 | 12 | | | |
| a | 0.222 | 0.625 | aa | 6 (1.245) | 4 (4.565) |
| | | | ab | 0 (4.245) | 5 (4.565) |
| | | | ac | 0 (2.264) | |
| | | | ad | 0 (2.717) | 1 (0.652) |
| | | | ae | | 1 (0.652) |
| b | 0.370 | 0.292 | bb | 9 (3.585) | 1 (0.913) |
| | | | bc | 0 (3.774) | |
| | | | bd | 2 (4.528) | 0 (0.304) |
| | | | be | | 0 (0.304) |
| c | 0.185 | 0 | cc | 4 (0.849) | |
| | | | cd | 2 (2.264) | |
| d | 0.222 | 0.042 | dd | 4 (1.245) | 0 (0) |
| | | | de | | 0 (0.430) |
| e | 0 | 0.042 | ee | | 0 (0) |

**Table 8.3** Observed (and expected) genotype frequencies of GPDH and 6GPDH in Colombia and Venezuela populations of *Lu. evansi*.

| | Colombia | | Venezuela | |
|---|---|---|---|---|
| Enzyme/genotype | Observed | Expected | Observed | Expected |
| GPDH | | | | |
| aa | 6 | 1.667 | 1 | 1.667 |
| ab | 3 | 11.667 | 13 | 11.667 |
| bb | 23 | 18.667 | 18 | 18.667 |
| Total | 32 | 32 | 32 | 32 |
| 6GPDH | | | | |
| aa | 9 | 6.840 | 5 | 2.129 |
| ab | 1 | 5.32 | 2 | 6.194 |
| ac | 3 | 0.840 | 0 | 1.548 |
| bb | 0 | 0 | 7 | 3.871 |
| bc | 0 | 0 | 0 | 2.065 |
| cc | 0 | 0 | 2 | 0.194 |
| Total | 13 | 13 | 16 | 16 |

**Table 8.4** Grouped frequencies of genotypes of GPDH and 6GPDH in Colombia and Venezuela populations of *Lu. evansi*.

| | | | Combined | |
|---|---|---|---|---|
| Enzyme/genotype | Colombia | Venezuela | Observed | Expected |
| GPDH | | | | |
| aa & ab | 9 | 14 | 23 | 26.668 |
| bb | 23 | 18 | 41 | 37.334 |
| Total | 32 | 32 | 64 | 64 |
| 6GPDH | | | | |
| aa | 9 | 5 | 14 | 8.969 |
| ab & bb | 1 | 9 | 10 | 15.385 |
| other | 3 | 2 | 5 | 4.647 |
| Total | 13 | 16 | 29 | 29 |

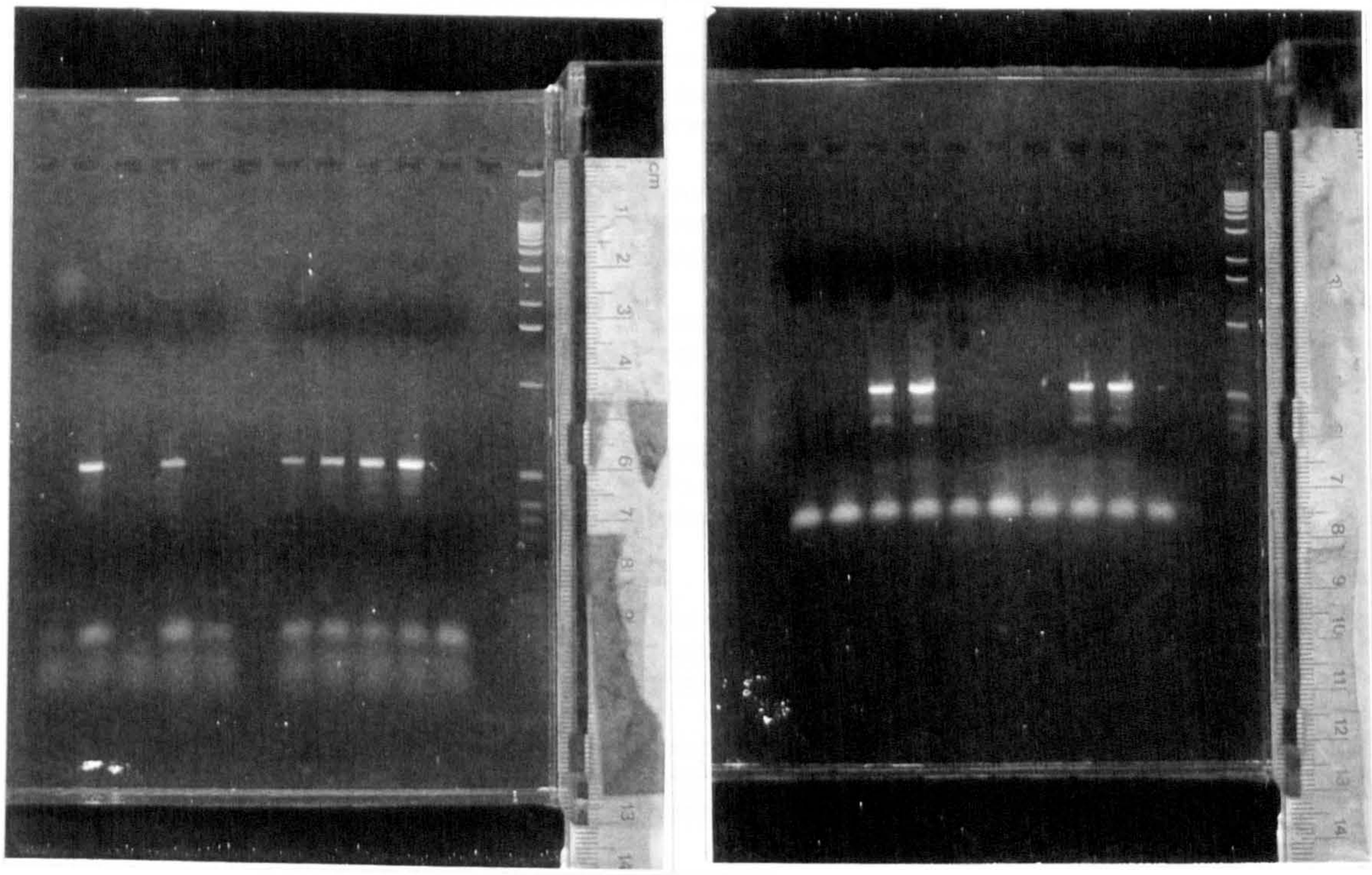


**Figure 8.2**. Bands of DNA revealed by agarose electrophoresis gel: C= Colombia; V= Venezuela; CR= Costa Rica; N= Nicaragua; CT= Control.

region while the remaining 3% were found in the first intergenic spacer (10bp). No base pair variations were found in the transfer RNAserine gene (UCN) or in the second intergenic spacer (Figure 8.3).

As expected, *Lu. whitmani* from Brazil, was distinct from the three *Lu. evansi* populations. The uncorrected pairwise dissimilarity distance between *Lu. evansi* and the standard *Lu. whitmani* sequence averaged 0.184. This was higher in Costa Rica (0.188) than both Colombia and Venezuela (0.183) (Table 8.5).

PAUP analysis of the 87 variant characters found among the four compared populations revealed that evanVE and evanCO are sister populations within a clade distinct from evanCR. Thus, two well defined clades are formed by *Lu. evansi* populations studied (Figure 8.4).

```
CO      CGACCAATTCCAAATAAATTAGGGGGAGTCATTGCCTTAGTCATATCAAT
VE      CGGTCAATTCCAAATAAATTAGGGGGAGTCATTGCCTTAGTAATATCAAT
CR      CGATCAATTCCAAATAAATTAGGAGGAGTAATTGCCTTAGTAATATCAAT
          **                  *     *           *
CO      TGCAATTTTATTCTTACTCCCTATTCTTCATATAAGAAAAATACAAGGAC
VE      TGCAATTTTATTCTTACTCCCTATTCTTCATATAAGAAAAATACAAGGAC
CR      TGCAATCCTATTTTTACTCCCTATCCTCCATATAAGAAAAATACAAGGCC
           **    *           *  *                      *
CO      TACAATTTTATCCTTTAAATCAAATTCTATTTTGATATATATTAATTATT
VE      TACAATTTTATCCTTTAAATCAAATTTTATTTTGATATATGTTAATTATT
CR      TACAATTTTATCCCTTAAATCAAATTTTATTCTGATATATATTAATTATT
                 *              *    *        *
CO      GTTATCTTATTAACATGAATTGGAGCCCGACCTGTAGAAGACCCCTATAT
VE      GTTGTCTTATTAACATGAATTGGAGCCCGGCCTGTAGAAGACCCCTATAT
CR      GTTATCCTATTGACATGAATTGGAGCCCGGCCAGTTGAAGATCTCTATAT
          * *    *                  *  *  *     * *
CO      TTTAACTGGGCAAATCCTAACTGTTATTTACTTCTTATATTATATTATCA
VE      TTTAACTGGACAAATCCTAACTGTTATTTACTTCTTATACTATATTATCA
CR      TTTAACAGGTCAAATTCTAACTGTTATTTATTTCTTATATTATATTATTA
           *  *     *                *         *        *


CO      ACCCATTAATTTCTAAAATTTGAGATAAAACTTTAAATTAATAAATTATT
VE      ACCCATTAATTTCTAAAATTTGAGATAAAACTTTAAATTAATAAATTATT
CR      ATCCATTAATCTCTAAAATTTGAGATAAAATTTTAAATCAATAAAATATT
         *        *                  *       *      *
CO      TAGTTAATAAGCTTTAATAGCAATTGTTTTGAAAACATTAGATAGAAACT
VE      TAGTTAATAAGCTTTAATAGCAATTGTTTTGAAAACATTAGATAGAAACT
CR      TAGTTAATAAGCTTTAATAGCAATTGTTTTGAAAACATTAGATAGAAACT


CO      CAAATTTTCTATTAACTTTACTAAAAATAATTAT
VE      CAAATTTTCTATTAACTTTACTAAAAATAATTAT
CR      CAAATTTTCTATTAACTTTACTAAAAATAATTAT
```

**Figure 8.3**. DNA sequence of PCR products obtained from *Lu. evansi* populations (CO= Colombia;VE= Venezuela and CR= Costa Rica). Stars indicate the pair-base differences.

**Table 8.5** Absolute (lower triangle) and mean (upper triangle) pairwise distances among *Lu. evansi* populations (evanCO= Colombia; evanVE= Venezuela and evanCR= Costa Rica) compared with *Lu. whitmani* (whitIL).

| | whitIL | evanVE | evanCO | evanCR |
|---|---|---|---|---|
| whitIL | ---- | 0.183 | 0.183 | 0.188 |
| evanVE | 71 | ---- | 0.023 | 0.077 |
| evanCO | 71 | 9 | ---- | 0.077 |
| evanCR | 73 | 30 | 30 | ---- |

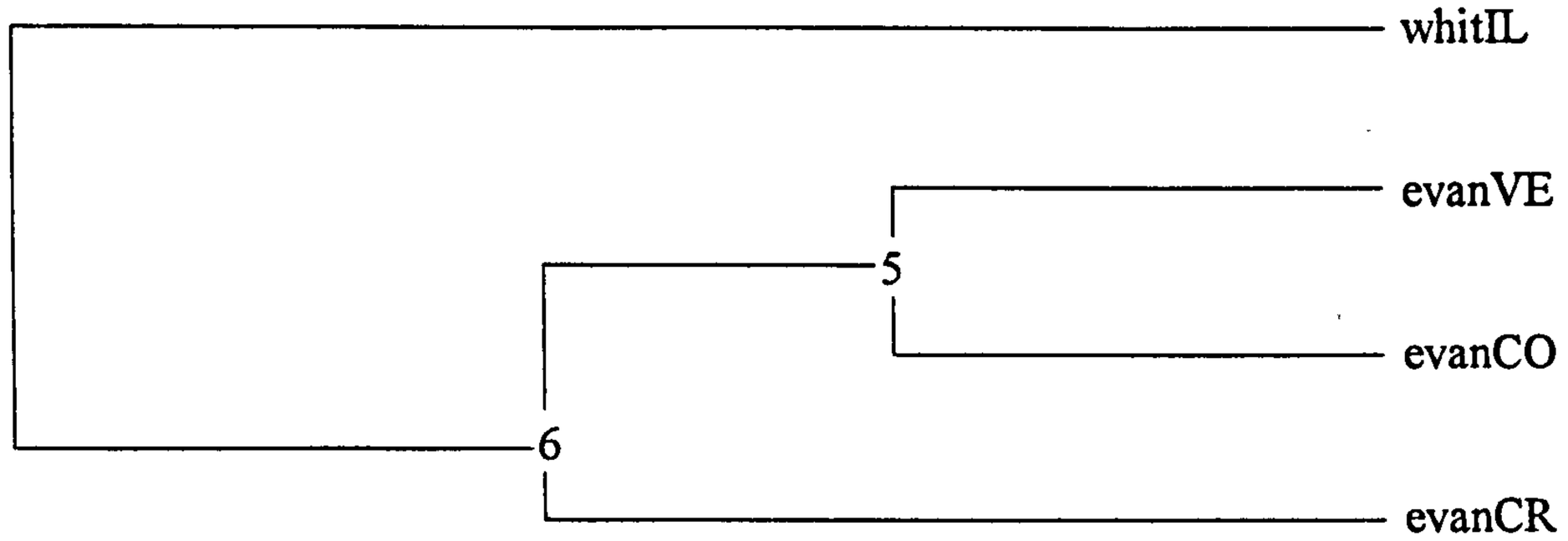


**Figure 8.4** Cladogram of relationships between three *Lu. evansi* populations (evanCO= Colombia; evanVE= Venezuela and evanCR= Costa Rica) and with *Lu. whitmani* (whitIL), inferred by PAUP from mtDNA sequences (Tree length = 95 steps, consistency index excluding uninformative characters = 1.0; retention index = 1.0).

## 8.4 Discussion

None of the *Lu. evansi* populations could be differentiated by traditional morphological analysis, and isozyme and mtDNA analysis showed the Colombia and Venezuela populations to be indistinguishable. However, mtDNA data did differ between Colombia and Venezuela (as a group) and Costa Rica. Ready et al. (submitted) have found mtDNA differences of the same order for sandfly morphospecies. Pairwise dissimilarities, as might be expected, are consistent with genetic distance correlated with geographic distance. An unbiased Nei identity index (Nei, 1978) of 0.92 suggests that the disequilibrium found in GPDH, PGM and 6GPDH is most likely regional, due to the geographical isolation of the species rather than a diagnostic difference within a single taxon. However, distances between sampling sites in Colombia and Venezuela are great

enough to preclude much gene flow between these populations given the assumption that *Lutzomyia* species are weak flyers, with limited dispersal capacity (Chianotis et al. 1974; Alexander & Young; 1992; Alexander, 1987; Morrison et al. 1993; present study). Either this assumption is wrong or there is considerable introgression between distant populations through neighbouring populations. The isozyme differences we observed between *Lu. evansi* populations are much smaller than those between the cryptic species *Lutzomyia* (*Psychodopygus*) *carrerai* and *Lu.(Ps.) yucumensis* (Caillard et al., 1986) and even within populations of *Lu. trapidoi* (Dujardin et al., 1996) but they are similar to the low polymorphism seen between close populations of *Lu. longipalpis* in Bolivia (Bonnefoy et al., 1986).

Unfortunately, the smallness of our samples from Central America, despite repeated attempts to collect more, limited both morphological and molecular approaches. The Costa Rican sample was based on spermathecal identification only. This is infortunate because we could have had a geological event in the development of the isthmus of Panama, some 3 million years ago (Hallam, 1994), to correlate with genetic differences between sandfly populations.

In conclusion, although PAUP analysis based on mtDNA data indicated a split in the *Lu. evansi* taxon, the question of the specific status of *Lu. evansi* remains unresolved. More data on geographical variation in biology, behavioural and reproductive characters are needed to determine if the two 'species' are reproductively isolated. This is a significant point to resolve because cryptic species could have distinct behaviours and consequently different vectorial capacities (Steiner, 1981).

# CHAPTER 9

# GENERAL DISCUSSION AND CONCLUSIONS

This chapter deals with the overall interpretation of the entomological and parasitological studies (experimental and observational) aimed at clarifying the role of *Lutzomyia evansi* (Núñez-Tovar) as a vector of *Leishmania chagasi*.

## The new American Visceral Leishmaniasis scenario

Five years have elapsed since the first report of *Lu. evansi* naturally infected with *L. chagasi* (Travi et al. 1990) in the San Andrés de Sotavento (SAS) area. During this time, primarily through this thesis, the sandfly fauna survey in this area was characterised by the total absence of *Lu. longipalpis* and the dominance of *Lu. evansi* in all collections (Table 3.1). New cases of visceral leishmaniasis were reported in the area during the study period. Biological, ecological and behavioural information has supported the hypothesis that in the visceral leishmaniasis (VL) focus of San Andrés de Sotavento, northern Colombia, *Lu. evansi* transmits *L. chagasi* to the exclusion of *Lu. longipalpis*. Furthermore, the collected data have elucidated the relationship between various entomological and epidemiological variables so that the most likely scenario for AVL in the Caribbean coast of Colombia can be deduced.

### Abundance and Seasonality

*Lu. evansi* has a bimodal seasonal abundance being, most common after rains (Figure 3.2). It also has a wide local and regional distribution (Tables 3.2 and 3.3). These two factors, distribution and abundance, could make *Lu. evansi* a primary candidate for interepidemic (ie. hypoendemic transmission) and the maintenance of *L. chagasi* during both dry and wet seasons. Certainly, the highest rates of infection have been reported three or four months after the occurrence of *Lu. evansi* peaks (B. Travi, WHO final report). *Lu. evansi* does not occur

or is present in very low numbers in some neighbouring places (Coveñas, Chinú, Sahagun, Sincelejo) where visceral leishmaniasis does not occur and *Lu. longipalpis* is absent also.

Domestication of the vector

As stated earlier (Chapter 2, section 2.1.4) when the position of individual VL cases in SAS are mapped, it appears that the majority live in houses in the forest area, ie. at the periphery of the village. At first sight this seems to contradict the results that the highest sandfly capture rates were reported in the open area (Figure 3.2). The first point to bear in mind is that in SAS area, changes in land use obscure the division between forest and open areas, therefore our definitions given for open and forest areas (section 2.2.1), were pragmatic and not all clear cut in biological terms. For example the differences are not as great as can be seen in wet tropical forest between cleared and uncleared areas. Although the highest densities of flies were recorded in the open area, overall the greatest number of parous flies was collected in the forest area, suggesting that hosts in the latter area have a much greater chance of contact with older flies (presumably with high potential of transmission). This is not surprising given the natural characteristic of gravid and fed flies to remain close to the patches of vegetation for resting and, probably, for breeding sites. Unfortunately, a limitation of this study was the inability to determine parity accurately in flies, especially in blood-fed flies. Consequently, their actual physiological status could not be estimated directly but only inferred from the rest of sandflies (unfed) caught on animals baits as well as from other sampling methods (eg. human bait landing rate, Shannon trap). A second factor favouring the high number of cases in forest may be related to the higher number of dogs observed in this area, also houses situated in this environment will probably have a higher activity of opossums (a wild reservoir) in peridomiciliary settings than those in open areas (Dr. G. Adler, pers. comm.). A combination of all the above factors would increase the chances of parasite transmission taking place more often in forest margins.

Whatever the area where the highest transmission rate occurs, it is important to point out the adaptability of *Lu. evansi* to both peri and intradomiciliary habitats (forest or open area) (Figures 3.5 and 3.6). The significance of *Lu. evansi* being found in houses is that transmission probably takes place there. The fact that children are infected means that they must come into contact with the vector. As the children do not go into the forest at night, transmission must take place inside houses. This is borne out by the daily biting cycle of *Lu.*

*evansi* - it is most active in the early evening (Figure 4.9) when children <5 years old are asleep in their houses.

The flies, although endophagic, do not rest in houses but leave to rest in tree-trunks for bloodmeal digestion. House spraying would appear to offer limited opportunity for control.

Host/reservoir(s)-vector contact

Since in AVL, humans are end-hosts and dogs (and probably opossums) represent a source of parasites for flies, variable degrees of both anthropophily and zoophily can be expected in the vector population. To a great extent, the fitness of *Leishmania* transmission relies on the ecological interaction vector-reservoir/human, one of the most fundamental points in the epidemiology of any vector-borne disease. At one level, transmission depends on the chance of acquisition of an infected meal by the vector from a reservoir(s). Secondly, once it has reached maturation, parasite dissemination is governed by sandfly survival and the chance of the vector contacting a suitable host or reservoir. One of the important targets here was to assess and quantify the relative attractiveness of key hosts and the feeding patterns of *Lu. evansi*. Results were conclusively demonstrated that at low densities *Lu. evansi* is an eclectic feeder (Figure 4.5), but it has a marked preference for humans over either dogs or opossums when the sandfly population increases (Table 4.2), although this can be modified by host location rather than host size (Figure 4.6). Feeding success depends on the hosts' defensive behaviour (Table 4.2),Assuming that the human landing rates give a fair estimation of the real human/vector contact, transmission in humans occurs when *Lu. evansi* reaches its seasonal peaks (Figure3.2) ie. August, November or even July ; which are the most probable periods when transmission takes place in SAS.

How do we explain the relatively high infection rates reported in dogs and the apparently low attractiveness of these animals to *Lu. evansi*?. This apparent paradox may have an explanation common to several vector borne diseases. Severity of a disease (in this case (leishmaniasis) depends of the ability of the parasite to inhibit the defences of the host (sensu Ewald, 1983). Thus it is possible that infection in dogs in SAS is the result of multiple infective bites allowing parasites to undermine the dog's immunological defence system over a period of time*. At present, the author and colleagues are carrying out a study with dogs and opossums employing similar methodologies to Courtenay et al. (1994) to address this

* most dogs were poorly nourished and this could also be a significant factor.

important subject.

Sandfly survival

During the present study, no attempts were made to establish directly the actual survival of *Lu. evansi* in nature. However it was shown that under lab conditions, batches of wild caught females deprived of humid oviposition places survived for 14 days, bred flies lived even longer (20 days). Results, obtained from the mark-release-recapture study strongly suggest that *Lu. evansi* females survive at least 8 days in nature (presumably more than the minimum period for a fly to acquire, mature and transmit *Leishmania*) (Table 4.6).

Parasite development

Although we failed to culture and identify the parasites found in naturally infected *Lu. evansi*, based on previous isolations and characterization of *L. chagasi* achieved in the same area (Travi et al., in press) and since this parasite species is dominant in SAS, it is reasonable to suppose that the natural infections found were *L. chagasi*. In addition, we carried out experimental infections in wild *Lu. evansi* verifying its susceptibility to both amastigote and promastigote forms of *L. chagasi* (Tables 7.2). The relatively limited range of vector species parasitised by *L. chagasi* in comparison to its close relatives *L. infantum* in the Old World suggests that *L. chagasi* has only recently began to radiate into the New World sandflies (Section 7). This independent evidence supports the hypothesis that *L. chagasi* is in fact *L. infantum* introduced into the Americas in historical times.

Transmission of *L. chagasi* by *Lu. evansi*

In summary, in the light of all the above points the most likely scenario for the transmission of *L. chagasi* to humans is as follows. Sandflies emerge from their breeding sites in the forest and move to houses in search of blood meals (section 4) or mates. Nulliparous flies move further into the village than parous flies (few cases of VL occur in the village centre and most flies caught are nulliparous). After feeding in houses flies leave and move to resting sites on tree-trunks, either trees around houses or trees in the forest (Section 5). Gravid flies and even fewer parous flies are found in the village centre after

oviposition. The newly parous flies move to the periphery of the village, perhaps the same night. This biting at the village periphery by parous flies increases the chances of transmission to the inhabitants. The increased number of dogs and opossums at the interface between the forest and the village also increases the chances of flies acquiring a *Leishmania* infection.

### Specific status of *Lu. evansi*

Whether *Lu. evansi* is a single species or represents a complex of at least two cryptic species is an open question. The results of the mtDNA studies suggest some degree of variation within this taxon (Table 8.5). More data are needed, particularly from Central American populations to resolve this apparent heterogeneity. If *Lu. evansi* is composed of more than one species then it is quite possible that the component species will have distinct behaviour and their vectorial capacity could be quite different. Therefore cautions must be taken in future in generalising from studies on one population.

## Perspectives for the control of American Visceral Leishmaniasis in San Andrés de Sotavento

Given the low incidence of VL and the low natural infection rates in *Lu. evansi* in the Caribbean Coast of Colombia it is tempting to suggest that the disease will resolve itself. However, this is highly unlikely because the features of this focus are typical of Old World hypoendemic VL foci due to *L. infantum*. The disease needs to be controlled and the appropriate approach- vector control , reservoir control or rapid case detection and treatment- cannot be determined without understanding the basic entomological, parasitological and sociological variables.

Several approaches to control are available. The parasite population could be affected by killing infected dogs accompanied by active detection and treatment of patients. However, sociological work is required to determine if these measures are acceptable. Since indians find any relationship between human and dog diseases, difficult to comprehend dog killing may not totally be accepted. Conventional treatment using pentavalent antimony injections in children has not been successful among aboriginal indians, who are usually suspicious of medical personnel and prefer local healers to treat

and cure their patients.

Although the present thesis give insights into the life history of *Lu. evansi* (eg. peridomiciliary and intradomiciliary habits, endophagic and exophilic behaviour, etc) some basic population parameters on this sandfly wait to be defined clearly (eg. survival rate). However there are sufficient findings to make suggestions for the control of VL in SAS. For instance, the facts that *Lu. evansi* is an eclectic feeder, it is apparently associated with some tree species to provide appropriate microecological conditions, and the possibility of dormancy in this species might be used in designing a rational control strategy. Zooprophylaxis added to the spraying of favoured resting places on trees during periods of sandfly dormancy might render good results.

As an alternative approach, and bearing in mind that though *L. chagasi* does not produce high mortality its prevalence is high in dogs and perhaps in opossums too and we have no evidence of human-human transmission, a main goal of *L. chagasi* control should be to prevent the link with humans. Infection in dogs (and probably in opossums) appears to be cumulative and of a long-term nature, therefore the continuous use of impregnated bednets (at least in children population to deplete both vector and parasite populations at the same time would be useful. Obviously, this plan requires plenty of sociological work before starting to guarantee acceptability by locals.

# REFERENCES


Alexander JB, 1987. Dispersal of phlebotomine sand flies (Diptera: Psychodidae) in a Colombian coffee plantation. **Journal of Medical Entomology 24**: 552- 558.

Alexander JB & Young DG, 1992. Dispersal of Phlebotomine sand flies (Diptera:Psychodidae) in a Colombian focus of *Leishmania* (*Viannia*) *braziliensis*.**Memórias do Instituto Oswaldo Cruz 87**: 397-403.

Alexander JB, Usma MC, Cadena H, Quesada BC, Solarte Y, Roa W, Montoya J, Jaramillo C & Travi BL, 1995. Phlebotomine sandflies associated with a focus of cutaneous leishmaniasis in Valle del Cauca, Colombia. **Medical & Veterinary Entomology 9**: 273-278.

Añez N, Nieves E & Cazorla D, 1989. The validity of the developmental pattern in the sandfly gut for classification of *Leishmania*. **Transactions of the Royal Society of Tropical Medicine & Hygiene 83**: 634-635.

Arciniegas Y & Duarte R, 1976. Kala-azar. **Tribuna Médica (Bogotá) 54**:38-39.

Ashford RW & Bettini S. 1987. Ecology and epidemiology: Old World. In: W Peters & RB Killick-Kendrick (Eds). **The Leishmaniases in Biology and Medicine.** Academic Press: New York.

Ashford RW, Desjeux P & deRaadt P, 1992. Estimation of population at risk of infection and number of cases of leishmaniasis. **Parasitology Today 8**: 104-105.

Asimeng EJ, 1991. Preliminary account of the phenology of some Nigerian savanna sandflies (Diptera: Psychodidae). **Journal of Medical Entomology 28**: 578-580.

Ayala MAR, Bergoxc PM & Anunciação EM, 1980. Calazar (primero caso autóctone no Sudeste do Paraná). **Journal Brasileiro de Medicina (Rio de Janeiro) 39:** 88-89.

Basimike M, Mutinga M & Kumar R, 1991. Distribution of sandflies (Diptera: Psychodidae) in three vegetation habits in the Marigat Area, Baringo District, Kenya. **Journal of Medical Entomology 28**: 330-333.

Barreto MP, 1941. Sobre a astenobiose do *Phlebotomus whitmani* Antunes e Coutihno, 1939 (Diptera: Psychodidae). **Revista Brasileira do Biologia (São Paulo). 1**: 305-310.

Beard CB, Mills Hamm D & Collins FH, 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. **Insect Molecular Biology 2**: 103-124.

Blanco-Tuirán PJ, Maingon RD, Hommel M, Alcalá JE, 1993. A focus of visceral and cutaneous leishmaniasis on the northern coast of Colombia. **Archives de l'Institut Pasteur de Tunis 70**: 481-488.

Bonnefoy S, Tybayrenc M, Le Pont F, Dujardin JP, Desjeux P & Ayala FJ, 1986. An isozymic study of *Lutzomyia longipalpis* (Diptera: Psychodidae), vector of visceral leishmaniasis in the 'Yungas' (Bolivia). **Cahiers ORSTOM series Entomologie Medicale et Parasitologie 24:** 213-217.

Borovsky D & Schlein Y, 1987. Trypsin and chemotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their role in vector competence. **Medical & Veterinary Entomology 1**: 747-755.

Bray RS, 1982. The zoonotic potential of reservoirs of leishmaniasis in the Old World. **Ecology & Disease 1**: 257-267.

Caillard T, Tybayrenc, Le Pont F, Dujardin JP, Desjeux P & Ayala FJ, 1986. Diagnosis by isozyme methods of two cryptic species, *Psychodopygus carrerai* and *P. yucumensis* (Diptera: Psychodidae). **Journal of Medical Entomology 23:** 489-492.

Camacho-Sánchez M, 1978. The distribution of kala-azar in Colombia, South America.**Transactions of the Royal Society of Tropical Medicine & Hygiene 72**: 544-545.

Camacho M, Caraballo LR, Barrios H, Correa I & Figueroa N, 1977. Kala-azar, un foco en el departamento de Sucre. **Tribuna Médica (Bogotá) 56**: 33-34.

Cameron MM, Milligan PJM, Llanos-Cuentas A & Davies CR. 1995. An association between phlebotomine sandflies and aphids in the Peruvian Andes. **Medical & Veterinary Entomology 9:** 127-132.

Cameron MM, Davies CR, Monje J, Villaseca P, Ogusuku E & Llanos-Cuentas A. 1994. Comparative activity of phlebotomine sandflies (Diptera: Psychodidae) in different crops in the Peruvian Andes. **Bulletin of Entomological Research 84**: 461- 467.

Cantillo J, Toro G, Saravia J, Calderón H & Mendoza C, 1970. Leishmaniasis visceral (Kala-azar) en Colombia. **Revista Latinoamericana de Patología 9**: 163-171.

Carbonell D, 1938. **Parasitología en Venezuela y los trabajos del doctor M. Núñez Tovar.** Litografía del Comercio: Caracas.

Cat I, Luz E, Borba AAm, Cassilha A, Costa P & Martins FL, 1974. Leishmaniose visceral autóctone de Minas Gerais. **O Hospital (Rio de Janeiro) 50**: 373-392.

Chagas E, Cunha AM, Castro GO, Ferreira LC & Romaña C, 1937. Leishmaniose visceral americana. Relatório dos trabalhos realizados pela Commissão encarregada do estudo sobre a leishmaniose visceral americana em 1936. **Memórias do Instituto Oswaldo Cruz 32**: 321-389.

Chagas E, Cunha AM, Ferreira LC, Deane L, Deane G, Guimaraes FN, Paumgartten MJ & Sá B, 1938. Leishmaniose visceral americana. Relatório dos trabalhos realizados pela Commissão encarregada do estudos da leishmaniose visceral americana em 1937. **Memórias do Instituto Oswaldo Cruz 33**: 89-229.

Chang KP, Fong D & Bray RS, 1985. Biology of *Leishmania* and leishmaniasis. In: KP Chang & RS Bray (Eds). **Leishmaniasis. Human Parasitic Diseases Series vol 1.** Elsevier: Oxford.

Chaniotis BN & Anderson JA, 1968. Age, structure, population dynamics and vector potential of *Phlebotomus* in northern California. Part II. **Journal of Medical Entomology 5**: 273- 292.

Chaniotis BN, Correa MA, Tesh RB & Johnson KM, 1974. Horizontal and vertical movements of phlebotomine sandflies in a Panamanian rain forest. **Journal of Medical Entomology 11**: 369- 375.

Chippaux JP, Pajot FX & Barbier D, 1984. Leishmaniasis in French Guyana 5. Further data on the ecology of the vector in the cacao forest village. **Cahiers ORSTOM Serie Entomologie Medicale et Parasitologie 22**: 213-218.

Christensen HA & Herrer A, 1980. Panamanian *Lutzomyia* (Diptera:Psychodidae) host attraction profiles. **Journal of Medical Entomology 17**: 522- 528.

Christensen HA & Herrer A, 1973. Attractiveness of sentinel animals to vectors of leishmniasis in Panama. **American Journal of Tropical Medicine & Hygiene 22**: 578-584.

Christensen HA, Fairchild GB, Herrer A, Johnson CM, Young DG & de Vasquez AM, 1983. The ecology of cutaneous leishmaniasis in the Republic of Panama. **Journal of Medical Entomology 20**: 463-484.

Clements AN, 1992. **The biology of mosquitoes. Vol 1**: Development, nutrition and reproduction. Chapman & Hall: London.

Corredor A, Ronderos TM & Rey M, 1980. Leishmaniasis visceral americana. Parte I. **Boletin Epidemiológico Nacional (Bogotá) 6:** 19-27.

Corredor AA, Osorno ME & Parra J, 1971. Contribucción al estudio epidemiológico del kala-azar

en Colombia. **Revista Facultad Medicina Universidad Nacional (Bogotá) 37**: 90-94.

Corredor AA, Pinzón MR, Cháparro CA, García HL & Zapata MT, 1986. Leishmaniasis tegumentaria americana. **Boletin Epidemiológico Nacional (Bogotá) 12**: 7- 52.

Corredor A, Gallego JF, Tesh RB, Morales A, De Carrasquilla FC, Young DG, Kreutzer RD, Boshell J, Palau MT, Cáceres E & Peláez D, 1989a. Epidemiology of visceral leishmaniasis. **American Journal of Tropical Medicine & Hygiene 40**: 480-486.

Corredor A, Gallego JF, Tesh RB, Peláez D, Díaz A, Montilla M & Palau MT, 1989b. *Didelphis marsupialis*, an apparent wild reservoir of *Leishmania donovani chagasi* in Colombia, South America.**Transactions of the Royal Society of Tropical Medicine & Hygiene 83**: 195.

Corredor A, Kreutzer RD, Tesh RB, Boshell J, Palau MT, Cáceres E, Duque S, Pélaez D, Rodríguez G, Nichols S, Hernández CA, Morales A, Young DG & De Carrasquilla FC, 1990. Distribution and etiology of leishmaniasis in Colombia. **American Journal of Tropical Medicine & Hygiene 42**: 206- 214.

Courmes E, Escudie A, Fauran P & Monnerville A, 1966. First native case of human visceral leishmaniasis in Guadeloupe. **Bulletin de la Societe Pathologie Exotique et de ses Filiales 59**: 217- 226.

Courtenay O, MacDonald DW, Lainson R, Shaw JJ & Dye C, 1994. Epidemiology of canine leishmaniasis: a comparative serological study of dogs and foxes in Amazon Brazil. **Parasitology 109:** 273-279.

Curtis CF & Rawlings P, 1980. A preliminary study of dispersal and survival of *Anopheles culicifacies* en relation to the possibility of inhibiting the spread of insecticide. **Ecological Entomology 5**: 11-17.

Danks HV, 1987. **Insect dormancy: an ecological perspective.** Biological Survey of Canada. Monograph series No 1. Ottawa: Canada.

D'Alessandro A, 1976. Biology of *Trypanosoma rangeli*. In: WHR Lumsden & DA Evans, (Eds.) **Biology of the kinetoplastida.** Academic Press: London & New York.

Davies CR, Cooper AM, Peacock C, Lane RP & Blackwell JM, 1990. Expression of LPG and GP63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. **Parasitology 101:** 337-343.

Day JF & Edman JD, 1984. Mosquito engorgement on normally defensive host depends on host activity patterns. **Journal of Medical Entomology 21**: 732-740.

Deane LM, Sarjeant SC & Fernandez E, 1978. Hallazgo de *Trypanosoma* (*Megatrypanum*)

*pessoai* Deane & Sugay, 1963, en murciélagos de Venezuela. **Boletin de la Dirección de Malariología y Saneamiento Ambiental (Caracas) 18**: 231- 237.

Desjeux P, 1992. Human leishmaniases: epidemiology and public health aspects. **World Health Statistics Quaterly 45**: 267- 275.

Dergacheva TI, Zherikhina II & Rasnitsyna NM, 1979. A method of counting sandflies (Diptera: Phlebotomidae). World Health Organisation, Unpubl. doc. WHO/Leish/ 79.15.

Dhanda V & Gill GS, 1982. Double blood meals by *Phlebotomus argentipes* and *Ph. papatasi* in two villages of Maharashtra. **Indian Journal of Medical Research 76**: 840-842.

Dillon RJ & Lane RP, 1993. Influence of *Leishmania* infection on blood-meal digestion in the sandflies *Phlebotomus papatasi* and *P. langeroni*. **Parasitological Research 79**: 492-496.

Doha S, Shehata G, El Said S & El Sawat B, 1991. Dispersal of *Phlebotomus papatasi* (Scopoli) and *P. langeroni* in El Hamman, Matrouh Governorate, Egypt. **Annales de Parasitologie Humaine et Comparee 66**: 69- 76.

Dujardin JP, Le Pont F, Cruz M, Leon R, Tarrieu F, Guderian R, Echeverria R & Tybayrenc M, 1996. Cryptic speciation in *Lutzomyia* (*Nyssomyia*) *trapidoi* (Fairchild & Hertig) (Diptera: Psychodidae) detected by multilocus enzyme electrophoresis. **American Journal of Tropical Medicine & Hygiene 54**: 42-45.

Dye C, 1994. Vector Control. In: ME Scott & G Smith (Eds). **Parasitic and Infectious Diseases.** Academic Press: London.

Dye C, Davies CR & Lainson R, 1991. Communication among phlebotomine sandflies: a field study of domesticated *Lutzomyia longipalpis* population in Amazonian Brazil. **Animal Behaviour 42**: 183- 192.

Elnaiem DA, Ward RD & Young PE, 1994. Development of *Leishmania chagasi* (Kinetoplastida: Trypanosomatidae) in the second blood-meal of its vector *Lutzomyia longipalpis* (Diptera: Psychodidae). **Parasitological Research 80:** 414- 419.

Elnaiem DA, Ward RD & Young PE, 1992. An ultrastructural study on the early development of *Leishmania chagasi* (Kinetoplastida: Trypanosomatidae) in its vector *Lutzomyia longipalpis* (Diptera: Psychodidae). **Annales de Parasitologie Humaine et Comparee 67:** 1-38.

El Sawaf BM, El Sattar SA, Shehata MG, Lane RP & Morsy TA, 1994. Reduced longetivity and fecundity in *Leishmania*-infected sand flies. **American Journal of Tropical Medicine & Hygiene 51:** 767-770.

El Sawaf BM, Mansour NS, El Said SM, Daba S, Yoursef FG, Kenawy MA & Beier JC, 1989.

Feeding patterns of *Phlebotomus papatasi* and *Phlebotomus langeroni* (Diptera: Psychodidae) in El Agamy, Egypt. **Journal of Medical Entomology 26**: 497-498.

Endris RG, Young DG & Perkins PV, 1987. Ultrastructural comparison of egg surface morphology of five *Lutzomyia* species (Diptera: Psychodidae). **Journal of Medical Entomology 24**: 413-415.

Espinal TLS & Montenegro ME, 1963. **Formaciones vegetales de Colombia**. Instituto Geográfico Agustin Codazzi: Bogotá.

Esterre P, 1986. Evaluation d'un programme de lutte contre la leishmaniose cutanee dans un village forestier de Guyane Francaise. **Bulletin World Health Organisation 64:** 559-565.

Ewald PW, 1983. Host-parasite relations, vectors, and the evolution of disease severity. **Annals Review of Ecology & Systematics 14:** 465-485.

Fausto AM, Maroli M & Mazzini M, 1991. Ootaxonomy investigation of three sandfly species (Diptera: Psychodidae) from Italy. **Parassitologia 33:** (Suppl. 1) 225- 228.

Feliciangeli DM, Arredondo C & Ward RD, 1992. Phlebotomine sandflies in Venezuela: Review of the *verrucarum* species group (in part) of *Lutzomyia* (Diptera: Psychodidae) with description of a new species from Lara. **Journal of Medical Entomology 29**: 729-744.

Feliciangeli MD, Castejon OC & Limongi J, 1993. Egg surface ultrastructure of eight New World phlebotomine sandfly species (Diptera: Psychodidae). **Journal of Medical Entomology 30**: 651-656.

Ferro C, Morrison AC, Torres M, Pardo R, Wilson ML & Tesh RB, 1995. Species composition and relative abundance of sand flies of the genus *Lutzomyia* (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. **Journal of Medical Entomology 32:** 527-537.

Foote BA, 1987. Order Diptera.In: F Stehr (Ed.). **Immature insects**. Kendall Hunt Publ. Co. Duboke: Iowa.

Foster WA, 1972. Studies on leishmaniasis in Ethiopia. III. Resting and breeding sites, flight behavior and seasonal abundance of *Phlebotomus longipes* (Diptera: Psychodidae). **Annals of Tropical Medicine & Parasitology 66**: 313- 328.

Foster WA, Boreham PFL & Tempelis CH, 1972. Studies on leishmaniasis in Ethiopia. IV: feeding behaviour of *Phlebotomus longipes* (Diptera: Psychodidae). **Annals of Tropical Medicine & Parasitology 66:** 433-443.

Fowler J & Cohen L, 1990. **Practical Statistics for Field Biology**. Open University Press:

Milton Keynes, Philadelphia.

Gallego J & Velez ID. 1995. Presencia en la Isla Fuerte, Bolivar, de *Lutzomyia evansi* vector de leishmaniosis visceral. **IATREIA (Medellin) 7**: 33-35.

García-Cuestas J, Pérez J, Franco G, Alvarado H & Bernal H, 1970. Tercer foco de Kala-azar en Colombia. **Tribuna Médica (Bogotà) 38**: 194.

Gast-Galvis A, 1944. Primer caso de leishmaniosis visceral en Colombia. **Anales de la Sociedad de Biología (Bogotá) 1**: 124- 125.

Gast-Galvis A & Renjifo S, 1944. Leishmaniosis visceral. Estudio epidemiológico del primer caso diagnosticado en Colombia. **Anales de la Sociedad de Biología (Bogotá) 1**: 1-8.

Ghosh KN & Bhatttacharya A, 1989. Laboratory colonization of *Phlebotomus argentipes* (Diptera: Psychodidae). **Insect Science & its Application 10**: 551-555.

Grimaldi GJr & Tesh RB, 1993. Leishmaniases of the New World: current concepts and implications for future research. **Clinical Microbiology Review 6**: 230-250.

Grimaldi GJr, David JR & McMahon-Pratt D, 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. **American Journal Tropical Medicine & Hygiene 36:** 270-287.

Grimaldi GJr, Tesh RB & McMahon-Pratt D, 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. **American Journal of Tropical Medicine & Hygiene 41**: 687-725.

Gómez-Vargas A, 1965. Leishmaniasis visceral en Colombia. Presentación de tres casos. **Antioquia Médica (Medellin) 15**: 323-324.

Guitton N & Sherlock IA, 1969. Descrição das fases imaturas do *Phlebotomus longipalpis* Lutz & Neiva, 1912 (Diptera: Psychodidae). **Revista Brasileira de Biologia (São Paulo) 29:** 383-389.

Hallam A, 1994. **An outline of Phanerozoic Biogeography.** Oxford Biogeography Series. Oxford University Press: Oxford.

Hanson WJ, 1968. The immature stages of the subfamily Phlebotominae in Panama. PhD Thesis Kansas University.

Hati AK, Tandon N, Sinha A, Sur S & De N, 1987. A comparative field study of some sandfly sampling methods. World Health Organisation. Unpub. doc. WHO/VBC/87.942.

Hebert PDN & Beaton MJ, 1989. **Methodologies for allozyme analysis using cellulose acetate electrophoresis. A practical handbook.** Univ. of Windsor: Ontario-Canada.

Hertig M & McConnell E, 1963. Experimental infection of Panamanian Phlebotomus sandflies

with *Leishmania*. **Experimental Parasitology 49:** 167- 174.

Hocking B, 1971. Blood-sucking behavior of terrestrial arthropods. **Annual Review of Entomology 16**: 1-26

Hopper KR, 1991. Ecological applications of elemental labelling: analysis of dispersal, density, mortality, and feeding. **Southwestern Entomologist 14:** 71-83.

Howard K, Kelly JM, Lane RP & Miles MM, 1991. A sensitive repetitive DNA probe that is specific to the *Leishmania donovani* complex and its use as an epidemiological and diagnostic reagent. **Molecular & Biochemical Parasitology 44**: 63-72.

Iversson LB, Camargo ME, Rocha E, Silva EO, Chieffi PP & Barros JAC, 1979. Investigação epidemiológica de um caso de leishmaniose visceral autóctone de Gran SãoPaulo. Brasil. **Revista de Saúde Pública (São Paulo) 13**: 159-167.

Jaramillo C, Travi BL & Montoya J, 1994. Vector competence of some Neotropical sandflies for the *Leishmania* (*Viannia*) *braziliensis* complex. **Medical & Veterinary Entomology 8**: 1-7.

Jeronimo SMB, Oliveira RM, Mackay S, Costa RM, Sweet J, Nascimento ET, Luz KG, Fernandes MZ, Jernigan J & Pearson RD, 1994. An urban outbreak of visceral leishmaniasis in Natal, Brazil. **Transactions of the Royal Society of Tropical Medicine & Hygiene 88**: 386-388.

Johnson RN, Ngumbi PM, Mwanyumba JP & Roberts CR, 1993. Host feeding preference of *Phlebotomus guggisbergi*, a vector of *Leishmania tropica* in Kenya. **Medical & Veterinary Entomology** 7: 216-218.

Johnson PT, McConnell E & Hertig M, 1963. Natural infections of leptomonad flagellates in Panamanian *Phlebotomus* species. **Experimental Parasitology 14:** 107-122.

Kay BH, 1979. Age structure of population of *Culex annulirostris* (Diptera: Culicidae) at Kowanyama and Charleville, Queesland. **Journal of Medical Entomology 16**: 309-316.

Kay BH, Boreham PFL & Williams GM, 1979. Host preferences and feeding patterns of mosquitoes (Diptera: Culicidae) at Kowanyama, Cape York Peninsula, northern Queesland. **Bulletin of Entomological Research 69**: 441-457.

Khan AA, Maibach HI & Strauss WG, 1971. A quantitative study of variation in mosquito response and host attractiveness. **Journal of Medical Entomology 8**: 41-43.

Killick-Kendrick R, 1990. Phlebotomine vectors of leishmaniasis: A review. **Medical & Veterinary Entomology 4**: 1-24.

Killick-Kendrick R, 1985. Some epidemiological consequences of the evolutionary fit between

leishmaniae and their phlebotomine vectors. **Bulletin de la Societe Pathologie Exotique et de ses Filiales 78:** 747- 755.

Killick-Kendrick R & Ward RD, 1981. Ecology of *Leishmania*. **Parasitology 82**: 143-152.

Killick-Kendrick R, Maroli M & Killick-Kendrick M, 1991. Bibliography on the colonization of Phlebotomine sandflies. **Parassitologia 33** (Suppl. 1): 321-329.

Killick-Kendrick R, Rioux JA, Bailly M, Guy, MW, Wilkes, TJ, Guy FM, Davidson I, Knechtli R, Ward RD, Guilvard E, Perieres J & Dubois H, 1984. Ecology of leishmaniasis in the south of France. 20. Dispersal of *Phlebotomus ariasi* Tonnoir, 1921 as a factor in the spread of visceral leishmaniasis in the Cévennes. **Annales de Parasitologie Humaine et Comparee 59**: 555- 572.

Killick-Kendrick R, Molyneux DH, Rioux JA, Lanotte G & Leaney AJ. 1980. Possible origins of *Leishmania chagasi*. **Annals of Tropical Medicine & Parasitology 74**: 563-565.

Killick-Kendrick R, Leaney AJ & Ready PD, 1977. The establishment, maintenance and productivity of a laboratory colony of *Lutzomyia longipalpis* (Diptera: Psychodidae). **Journal of Medical Entomology 13:** 429-440.

Klowden MJ & Lea AO, 1978. Blood meal size as a factor affecting the continued host-seeking in *Aedes aegypti* (L.) **American Journal of Tropical Medicine & Hygiene 27**: 827-831.

Kreutzer RD, Palau MT, Morales A, Ferro C, Feliciangeli D & Young DG. 1990. Genetic relationships among Phlebotomine sand flies (Diptera: Psychodidae) in the *verrucarum* species group. **Journal of Medical Entomology 27**: 1-8.

Kreutzer RD, Corredor A, Grimaldi Gjr, Grogl M, Rowton ED, Young DG, Morales A, McMahon-Pratt D, Guzman H & Tesh RB, 1991. Characterization of *Leishmania colombiensis* sp. n. (Kinetoplastida: Trypanosomatidae), a new parasite infecting humans, animals and phlebotomine sandflies in Colombia and Panama. **American Journal of Tropical Medicine & Hygiene 44:** 662-675.

Lainson R, & Shaw JJ, 1987. Evolution, classification and geographical distribution. In: W Peters & RB Killick-Kendrick (Eds). **The Leishmaniases in Biology and Medicine.** Academic Press: New York.

Lainson R, & Shaw JJ, 1979. The role of animals in the epidemiology of South American leishmaniasis. In: WHR Lumsden & DA Evans (Eds). **Biology of Kinetoplastida, Vol II.** Academic Press: London. 1-116.

Lainson R, Shaw JJ, Ryan L, Ribeiro RSM, & Silveira FT, 1985. Leishmaniasis in Brazil: XXI. Visceral leishmaniasis in the Amazon Region and further observations on the role of

*Lutzomyia longipalpis* (Lutz & Neiva, 1912) as the vector. **Transactions of the Royal Society of Tropical Medicine & Hygiene 79**: 223- 226.

Lainson R, Shaw JJ, Silveira FT, & Fraiha H, 1983. Leishmaniasis in Brazil: XIX. Visceral leishmaniasis in the Amazon Region, and the presence of *Lutzomyia longipalpis* on the Island of Marajó, Pará State. **Transactions of the Royal Society of Tropical Medicine & Hygiene 77**: 323- 330.

Lainson R, Ward RD & Shaw JJ, 1977. Experimental transmission of *Leishmania chagasi*, causative agent of neotropical visceral leishmaniasis, by the sandfly *Lutzomyia longipalpis*, **Nature 266**: 628- 630.

Lane RP, 1993. Sandflies (Phlebotominae). In: RP Lane & RW Crosckey (Eds). **Medical Insects and Arachnids**. Chapman and Hall: London.

Lane RP, Pile MM & Amerasinghe FP, 1990. Anthropophagy and aggregation behaviour of the sandfly *Phlebotomus argentipes* in Sri Lanka. **Medical & Veterinary Entomology 4**: 79-88.

Lawyer PG, Rowton ED, Perkins PV, Johnson RN & Young DG, 1991. Recent advances in laboratory mass rearing of Phlebotomine sand flies. **Parassitologia 33** (Suppl. 1): 361-364.

Lawyer PG, Young DG, Butler JF & Akin DE, 1987. Development of *Leishmania mexicana* in *Lutzomyia diabolica* and *Lutzomyia shannoni* (Diptera; Psychodidae). **Journal of Medical Entomology 24:** 347- 355.

Le Pont F & Desjeux P, 1985. Leishmaniasis in Bolivia. I. *Lutzomyia longipalpis* (Lutz & Neiva, 1912) as the vector of visceral leishmaniasis in Los Yungas. **Transactions of the Royal Society of Tropical Medicine & Hygiene 79**: 227- 231.

Lewis DJ, 1975. Functional morphology of the mouth parts in New World phlebotomine sandflies (Diptera: Psychodidae). **Transactions of the Royal Society of Entomology (London) 126**: 497-532.

Lewis DJ, 1974. The biology of Phlebotomine in relation to leishmaniasis. **Annual Review of Entomology 19**: 363-384.

Loyola EG, Alzate A, Sánchez A & González A, 1988a. Epidemiology of a natural focus of *Leishmania braziliensis* in the Pacific lowlands of Colombia. III. Natural infections in wild mammals. **Transactions of the Royal Society of Tropical Medicine & Hygiene 82**: 406-407.

Loyola EG, Alzate A, Sánchez A & González A, 1988b. Epidemiology of a natural focus of

*Leishmania braziliensis* in the Pacific lowlands of Colombia. IV. Observations on sandflies. **Transactions of the Royal Society of Tropical Medicine & Hygiene 82**: 408.

Lysenko AJ & Beljaev AE, 1987. Quantitative approaches to epidemiology. In: W Peters & RB Killick-Kendrick Eds. **The Leishmaniases in Biology and Medicine.** Academic Press: New York.

Magurran AE, 1988. **Ecological diversity and its measurement.** Croom Helm: London.

Marsden PD & Jones TC, 1985. Clinical manifestations, diagnosis and treatment of leishmaniasis. In: KP Chang & RS Bray (Eds). **The Leishmania.** Elsevier: New York. 183- 189.

Mayika K & Taguchi I, 1982. Ecological studies on overwintering populations of *Culex pipiens pallens*. 3. Movement of the mosquitoes in a cave during overwintering. **Japanese Journal of Sanitary Zoology 33**: 335-343.

Memmott J, 1992. Patterns of sandfly distribution in tropical forest: a causal hypothesis. **Medical &Veterinary Entomology 6:** 188-194.

Miles M & Ward RD, 1978. Preliminary isoenzyme studies on Phlebotomine sandflies (Diptera: Psychodidade). **Annals of Tropical Medicine & Parasitology 72:** 398-399.

Mirsa A, 1953. Sobre la biologia de algunos flebótomos (Díptera: Psychodidae) y datos sobre otros hematófagos colectados en Altagracia de Orituco (Estado Guárico, Venezuela). **Revista de Sanidad & Asistencia Social (Caracas) 17**:189-196.

Modi GB & Tesh RB, 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidade) in the laboratory. **Journal of Medical Entomology 20**: 568-569.

Molyneux DH, 1983. Host-parasite relationships of Trypanosomatidae in vectors. In: KF Harris. (ed). **Current Topics in Vector Research, Vol. 1.** Praeger Publ. New York.

Montoya-Lerma J & Lane RD, 1996a. Factors affecting host attractiveness and feeding behaviour of *Lutzomyia evansi* (Diptera: Psychodidae), a vector of visceral leishmaniasis in Colombia. **Bulletin of Entomological Research 86**: 43-50.

Montoya-Lerma J & Lane RD, 1996b. An efficient and economic trap to collect sandflies around hosts. **Bulletin of Entomological Research** (In press).

Montoya J, Jaramillo C, Palma G, Gómez T, Segura I & Travi B, 1990. Report of an epidemic outbreak of tegumentary leishmaniasis in a coffee-growing area of Colombia. **Memórias do Instituto Oswaldo Cruz 85**: 119-121.

Montoya-Lerma J, Cadena H, Arroyo C & Travi BL, *Lutzomyia columbiana* (Diptera: Psychodidae) vector of *Leishmania mexicana* in Colombia (In prep.).

Morales A, Corredor A, Cáceres E, Ibagos E & Rodriguez CI, 1981. Aislamiento de tres cepas de *Leishmania* a partir de *Lutzomyia trapidoi* en Colombia. **Biomédica (Bogotá): 5**: 129-138.

Moreno G & Oviedo M, 1995. Bionomy of vector of American visceral leishmaniasis in Trujillo State, Venezuela. II. Longitudinal study of *Lutzomyia evansi* in endemic situation. II Simposio Internacional sobre Flebotomineos. (ISOPS II) Merida- Venezuela. **Talleres 4:** 66.

Morris RF, 1960. Sampling insect populations. **Annual Review of Entomology 5:** 243- 264.

Morrison AC, 1994. Field studies on the ecology of the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) at an endemic focus of American visceral leishmaniasis in Colombia. Ph.D. Thesis. Yale University.

Morrison AC, Ferro C & Tesh RB, 1993a. Dispersal of the sand fly *Lutzomyia longipalpis* at an endemic focus of American visceral leishmaniasis in Colombia. **Journal of Medical Entomology 30**: 427-435.

Morrison AC, Ferro C & Tesh RB, 1993b. Host preferences of the sand fly *Lutzomyia longipalpis* at an endemic focus of American visceral leishmaniasis in Colombia. **American Journal of Tropical Medice & Hygiene 49**: 68-75.

Mukhopadhyay AK, Kumar K & Rahman SJ, 1987. Host preference of phlebotomine sandflies in and around Delhi. **Journal of Communicable Diseases 19**: 408-411.

Munir MA, 1994. Systematics and biology of Phlebotomine sandflies of the visceral leishmaniasis foci of northern Pakistan. Ph.D. Thesis. University of London.

Mutinga MJ, Kyai FM, Kamau, C & Omogo DM, 1986. Epidemiology of leishmaniasis in Kenya. III. Host preference studies using various types of animal burrows in Marigat, Baringo District. **Insect Science & its Application 7**: 191-197.

Namita M, Joshi V & Bansal SK, 1991. Host preference pattern of phlebotomine sandflies of Bikaner city. **Indian Journal of Medical Research 93:** 328-329.

Navin TR, Sierra M, Custodio R, Steurer F, Porter CH & Trenton KRII, 1985. Epidemiologic study of visceral leishmaniasis in Honduras, 1975-1983. **American Journal of Tropical Medicine & Hygiene 34**: 1069-1075.

Nei M, 1978. Estimation of average heterorozygosity and genetic distance from a small number of individuals.**Genetics 89:** 583-590.

Osorno E, Morales A, Osorno F & Ferro C, 1972. Phlebotominae de Colombia IV. Distribucción geográfica de especies de *Brumptomyia* Fraca y Parrot, 1921 y *Lutzomyia* Fraca, 1924

encontrados en Colombia, S.A. **Revista de la Academia de Ciencias Exactas Fisicas & Naturales (Bogotá) 14**: 1- 81.

Oviedo M, Moreno G & Graterol D, 1995. Bionomy of vector of American visceral leishmaniasis inTrujillo State, Venezuela. III. Colonization of *Lutzomyia evansi*. II Simposio Internacional sobre Flebotomineos. (ISOPS II) Merida- Venezuela. **Talleres 4:** 79.

Pandya AP, 1980. Laboratory breeding of the sandflies *Phlebotomus argentipes* Annandale & Brunetti and *P. papatasi* Scopoli. **Indian Journal of Medical Research 72**: 64-65.

Petersen JL, 1982. Preliminary survey of isozyme variation in anthropophilic Panamanian *Lutzomyia* species. In: Chance ML & Walton BC (Eds). **Biochemical characterization of *Leishmania*** UNPD/ World Bank/WHO.

Petersen JL, 1984. Identification of phlebotomine sandflies (Diptera: Psychodidae) by cellulose acetate eletrophoresis. In: BN Newton & F Michal (ed). **New approaches to the identification of parasites and their vectors.** Schwabe & Co.: Basel. 349-362.

Pérez-Norsagaray J, García J & Franco G, 1970. Segundo foco endémico de Kala-azar en Colombia. **Tribuna Médica (Bogotà) 38**: 8-11.

Pifano F & Romero J, 1973. Comprobación de un foco autóctono de leishmaniasis visceral (kala-azar) en la isla de Margarita, Edo. Nueva Esparta, Venezuela. **Archivos Venezolanos de Medicina Tropical & Parasitología Médica 5**: 129-144.

Pifano F & Romero J, 1964. Investigaciones epidemiológicas sobre la leishmaniasis visceral en la Isla de Margarita, Edo., Nueva Esparta, Venezuela. **Gaceta Médica (Caracas) 5**: 427-430.

Pimenta PFP, Saraiva EMB, Rowton E, Modi GB, Garraway LA, Beverley SM, Turco SJ & Sacks DL, 1994. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. **Proceedings of the National Academy of Sciences of theUnited States of America 91:** 9155- 9159.

Pratlong F, Dedet JP, Marty P, Portús M, Deniau M, Dereure J, Abranches P, Reynes J, Martini A, Lefebvre M & Rioux JA, 1995. *Leishmania*-human immunodeficiency virus coinfection in the Mediterranean Basin: isoenzymatic characterization of 100 isolates of the *Leishmania infantum* complex. **Journal of Infectious Dieases 172**: 323-326.

Price PW, 1980. **Evolutionary Biology of Parasites. Monographs in Population Biology 15.** Princenton University Press, Princeton: New Jersey.

Quate LW, 1964. *Phlebotomus* sandflies of the Paloich area in the Sudan (Diptera: Psychodidae).

**Journal of Medical Entomology 1**: 213- 268.

Quinnell RJ & Dye C, 1994. An experimental study of peridomestic distribution of *Lutzomyia longipalpis* (Diptera: Psychodidae). **Bulletin of Entomological Research 84**: 379-382.

Quinnell RJ, Dye C & Shaw JJ, 1992. Host preferences of the phlebotomine sandfly *Lutzomyia longipalpis* in Amazonian Brazil. **Medical & Veterinary Entomology 6**: 195-200.

Rangel EF, de Souza NA, Wermenlinger ED & Barbosa AF, 1985. Estabelecimento de colonia, em laboratório, de *Lutzomyia intermedia* Lutz & Neiva, 1912 (Diptera: Psychodidae, Phlebotominae).**Memórias do Instituto Oswaldo Cruz 80:** 219-226.

Ready PD, 1979. Factors affecting egg production of laboratory-bred *Lutzomyia longipalpis* (Diptera: Psychodidae). **Journal of Medical Entomology 16**: 413-423.

Ready PD, Lainson R, Wilkes TJ & Killick-Kendrick R, 1984. On the accuracy of age-grading neotropical phlebotomines by counting follicular dilations: first laboratory experiments, using colonies of *Lutzomyia flaviscutellata* (Mangabeira) and *L. furcata* (Mangabeira) (Diptera: Psychodidae). **Bulletin of Entomological Research 74**: 641- 646.

Ready PD, Day JC, Souza AA, Rangel EF & Davies CR. Mitochondrial DNA characterization of populations of *Lutzomyia whitmani* (Diptera: Phlebotominae) incriminated in the peridomestic transmission of *Leishmania* species in Brazil (Submited).

Reeves WC, Brookman B & Hammon W McD, 1948. Studies on the flight range of certain *Culex* mosquitoes, using a fluorescent-dye marker, with notes on *Culiceta* and *Anopheles*. **Mosquito News 8:** 61-69.

Rioux JA, Perieres J, Killick-Kendrick R, Lanotte G & Bailly M,1982. Ecologie des leishmanioses dans le sud de la France.17. Enchantillonage des phlebotomes par le procede des pieges adhesifs. Comparison avec la technique de capture sur appat humain. **Annales de Parasitologie Humaine et Comparee 57**: 631-635.

Rioux JA, Killick-Kendrick R, Leaney AJ, Turner DP, Bailly M & Young CJ, 1979. Ecologie des Leishmanioses dans le sud de la France. 12. Dispersion horizontale de *Phlebotomus ariasi* Tonnoir, 1921. Expériences préliminaires. **Annales de Parasitologie Humaine et Comparee 54**: 673- 682.

Rodríguez G, Corredor A, Cáceres E, Cassiano G, Arroyo C, Palaú MT & Boshell J, 1985. Leishmaniasis difusa. **Biomédica (Bogotà) 5**: 95- 111.

Rosabal R & Trejos A, 1965. Phlebotomus de El Salvador (Diptera: Psychodidae) II. Observaciones sobre su biología con especial referencia a *P. longipalpis*. **Revista de Biología Tropical 13** 219- 228.

Ryan L, Lainson R, Shaw JJ, Braga RR & Ishikawa AY, 1987. Leishmaniasis in Brazil. XXV. Sandfly vectors of *Leishmania* in Pará State, Brazil. **Medical & Veterinary Entomology 1**: 383-395.

Ryan L, Silveira FT, Lainson R & Shaw JJ, 1984. Leishmanial infections in *Lutzomyia longipalpis* and *Lu. antunesi* (Diptera: Psychodidae) in the island of Marajó, Para, State, Brazil. **Transactions of the Royal Society of Tropical Medicine & Hygiene 78**: 547-548.

SAS Institute Inc, 1982. **Users guide: Statistics.** Cary, NC.

Saraiva EMB, Pimenta PFP, Brodin TN, Rowton E, Modi GB & Sacks DL, 1995. Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* in *Phlebotomus papatasi*. **Parasitology 111**: 275- 287.

Saravia NG, Holguín, AF, McMahon-Pratt D & D'Alessandro A, 1985. Mucocutaneous leishmaniasis in Colombia: *Leishmania braziliensis* subspecies diversity. **American Journal of Tropical Medicine & Hygiene 34**: 714- 720.

Service MW, 1993. **Mosquito Ecology. Field sampling methods. 2nd Ed.** Elsevier Applied Science: London- New York.

Service MW, Voller A & Bidwell DE, 1986. The enzyme-linked immunosorbent assay (ELISA) test for the identification of blood-meals of haematophagous insects. **Bulletin of Entomological Research 76**: 321- 330.

Shaw J & Lainson R, 1987. Ecology and epidemiology: New World. In: W Peters & RB Killick-Kendrick (Eds). **The Leishmaniases in Biology and Medicine.** Academic Press: New York.

Shaw JJ & Lainson R, 1968. Leishmaniasis in Brazil. II. Observations on enzootic rodent leishmaniasis in the lower Amazon region- the feeding habits of the vector, *Lutzomyia flaviscutellata* in reference to man, rodents and other animals. **Transactions of the Royal Society of Tropical Medicine & Hygiene 62**: 396- 405.

Shannon RC, 1939. Methods for collecting and feeding mosquitoes in jungle yellow fever studies. **American Journal of Tropical Medicine & Hygiene 19**: 131-138.

Sherlock IA & Miranda JC, 1993. Ecology of the visceral leishmaniasis in the State of Bahia, Brazil. In: SP Brandão-Filho (Ed.) **Research and control of leishmaniasis in Brazil. Proccedings of workshop held in Recife, Brazil** (September 1993): 133-149.

Southwood TRE, 1980. **Ecological Methods,** 2nd ed. Chapman & Hall: London.

Strangways-Dixon J & Lainson R, 1966. The epidemiology of dermal leishmaniasis in British

Honduras. Part III. The transmission of *Leishmania mexicana* to man by *Phlebotomus pessoanus*, with observations on the development of the parasite in different species of *Phlebotomus*. **Transactions of the Royal Society of Tropical Medicine & Hygiene 60**: 192- 207.

Steiner WWM, 1981. Parasitization and speciation in mosquitoes: a hypothesis. In: R Pal, Kitzmiller & T Kanda (Eds). **Cytogenetics and genetics of vector. Proceedings of a symposium of the XVIth Congress of Entomology**. Kodansha Ltd: Tokyo. Elsevier Biomedical.

Swofford DL, 1993. **PAUP: Phylogenetic Analysis Using Parsimony Version 3.1.** Computer Program distributed by the Illinois Natural History Survey. Champaing : Illinois.

Tesfa-Yohannes TM, 1982. Reproductive biology of *Aedes* (*S.*) *malayensis* (Diptera: Culicidae). **Journal of Medical Entomology 19**: 29-33.

Tesh RB, Chianiotis BN, Carrera BR & Johnson KM, 1972. Further studies on the natural host preferences of Panamanian phlebotomine sandflies. **American Journal of Epidemiology 95:** 88-93.

Tesh RB, Chianiotis BN, Aronson MD & Johnson KM, 1971. Natural host preferences of Panamanian sandflies as determined by precipitin test. **American Journal of Tropical Medicine & Hygiene 20:** 150-156.

Theodor O, 1965. On the classification of American Phlebotominae. **Journal Medical Entomology 2**: 171-197.

Travi BL, Jaramillo C, Montoya J, Segura I, Zea A, Gonçalves A & Vélez ID, 1994. *Didelphis marsupialis*, an important reservoir of *Trypanosoma* (*Sshizotrypanum*) *cruzi* and *Leishmania* (*Leishmania*) *chagasi* in Colombia. **American Journal of Tropical Medicine & Hygiene 50**: 557- 565.

Travi BL, Vélez ID, Brutus L, Segura I, Jaramillo C & Montoya J, 1990. *Lutzomyia evansi*, an alternate vector of *Leishmania chagasi* in a Colombian focus of visceral leishmaniasis. **Transactions of the Royal Society Tropical Medicine & Hygiene 84**: 676-677.

Travi BL, Montoya J, Solarte Y, Lozano L & Jaramillo C, 1988. Leishmaniasis in Colombia. I. Studies on the phlebotomine fauna associated with endemic foci in the Pacific coast region. **American Journal of Tropical Medicine & Hygiene 39**: 261- 266.

Travi BL, Montoya J, Gallego J, Jaramillo C, Llano R & Vélez ID, 1996. Bionomics of *Lutzomyia evansi* (Diptera: Psychodidae) vector of visceral leishmaniasis in northern Colombia. **Journal of Medical Entomology** (in press).

Varley GC, Gradwell GR & Hassell MP, 1975. **Insect population ecology**. Blackwell Scientific Publications: Oxford.

Velasco JE, 1973. The phlebotomine sandflies of the Las Yungas region of Bolivia. MSc Thesis. Department of Tropical Medicine & Medical Parasitology: Louisiana State University.

Voller A, Barttlet A & Bidwell DE, 1976. Enzyme immunoassays for parasitic diseases. **Transactions of the Royal Society of Tropical Medicine & Hygiene 70**: 98-106.

Voller A, Bidwell DE & Barttlet A, 1980. Enzyme linked immunosorbent assay. In: N Rose & H Friedman (Eds). **Manual of Clinical Immunology**. American Society of Microbiology 359-371: Washington.

Vélez ID, Ghysais G, Marulanda J, Maya DA, Rivera I, Guerrero MA, Hurtado MI, Cadavid JM, Ocampo A, Arbeláez B & Wolff M, 1988. Leishmaniasis tegumentaria americana encuesta epidemiológica en una comunidad indigena. **Iatreia (Medellin) 1**: 29- 33.

Walters LL, 1993. *Leishmania* differentiation in natural and unnatural sand fly hosts. **Journal of EukaryoticMicrobiology 40**: 196- 206.

Walters LL, Irons K, Modi GB, Chaplin GL & Tesh RB, 1992. Refractory barriers in the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae) to infection with *Leishmania panamensis*. **American Journal of Tropical Medicine & Hygiene 46: 211- 228.**

Walters LL, Chaplin GL, Modi GB & Tesh RB, 1989. Ultrastructural biology of *Leishmania (Viannia) panamensis (= Leishmania braziliensis panamensis)* in *Lutzomyia gomezi* (Diptera: Psychodidae): a natural host-parasite association. **American Journal of Tropical Medicine & Hygiene 40**: 19-39.

Walters LL, Modi GB, Chaplin GL & Tesh RB, 1989. Ultrastructural development of *Leishmania chagasi* in its vector *Lutzomyia longipalpis* (Diptera: Psychodidae). **American Journal of Tropical Medicine & Hygiene 41**: 295- 317.

Walters LL, Modi GB, Chaplin GL, Tesh RB & Burrage T, 1987. Host-parasite relationship of *Leishmania mexicana* and *Lutzomyia abonnenci* (Diptera: Psychodidae). **American Journal of Tropical Medicine & Hygiene 36**: 294- 314.

Warburg A, Montoya-Lerma J, Jaramillo C, Cruz-Ruiz AL & Ostrovska K, 1991. Leishmaniasis vector potential of *Lutzomyia* spp. in Colombian coffee plantations. **Medical & Veterinary Entomology 5:** 9-16.

Ward RD, 1972. Some observations on the biology and morphology of the immature stages of *Psychodopygus wellcomei* Fraiha, Shaw and Lainson, 1971. (Diptera: Psychodidae). **Memórias do Instituto Oswaldo Cruz 70:** 15-28.

Ward RD, 1976. A revised numerical chaetotaxy for neotropical Phlebotomine sandfly larvae (Diptera: Psychodidae). **Systematic Taxonomy 1**: 89-94.

Ward RD & Ready PA, 1975. Chorionic sculpturing in some sandfly eggs (Diptera: Psychodidae). **Journal of Entomology (A) 50:** 127-134.

Ward RD, Hamilton JGC, Dougherty M, Falção AL, Feliciangeli MD, Perez JE & Veltkamp CJ, 1993. Pheromone disseminating structures in tergites of male phlebotomines (Diptera: Psychodidae). **Bulletin of Entomological Research 83**: 437- 445.

Ward RD, Pasteur N & Rioux JA, 1981. Electrophorectic studies on genetic polymorphism and differentiation of phlebotominae sandflies (Diptera: Psychodidae) from France and Tunisia. **Annals of Tropical Parasitology 75:** 235-245.

Werner JK & Barreto P, 1981. Leishmaniasis in Colombia, a review. **American Journal of Tropical Medicine &Hygiene 30**: 751-761.

Williams P, 1970. Phlebotomine sandflies and leishmaniasis in British Honduras (Belize). **Transactions of the Royal Society of Tropical Medicine Hygiene 64:** 317- 368.

World Health Organization, 1990. Control of the Leishmaniases:**Technical Report Series 793** : 3-158.

World Health Organization, 1972. Vector Ecology. **Technical Report Series 501:** 1-38.

Young DG, 1979. **A review of the bloodsucking psychodid flies of Colombia (Diptera: Phlebotominae and Sycoracinae). Bulletin (techical) Agriculture Experimental Station University of Florida** 806.

Young DG & Duncan MA, 1994. Guide to identification and geographic distribution of *Lutzomyia* and sand flies in Mexico, West Indies, Central and South America (Diptera: Psychodidae). **Memoirs of the American Entomological Institute 54:** 1-881.

Young DG & Arias JR, 1991. Phlebotomine sandflies in the Americas. **Pan American Health Organization. (Tech. Paper) 33**: 1-26.

Young DG, Morales A, Kreutzer RD, Alexander JB, Corredor A, Tesh RB, Ferro de Carrasquilla C, & de Rodríguez, C, 1987. Isolation of *Leishmania braziliensis* (Kinetoplastida: Trypanosomatidae) from cryopreserved Colombian sandflies (Diptera: Psychodidae). **Journal of Medical Entomology 24**: 587-589.

Yuval B, 1991. Populations of *Phlebotomus papatasi* (Diptera: Psychodidae) and the risk of *Leishmania major* transmission in three Jordan Valley habitats. **Journal of Medical Entomology 28**: 492-495.

Zeledón R, Murillo J & Gutiérrez H, 1984. Observaciones sobre la ecología de *Lutzomyia longipalpis* (Lutz & Neiva, 1912) y posibilidades de existencia de leishmaniasis visceral en Costa Rica.**Memórias do Instituto Oswaldo Cruz 79**: 455-459.

Zeledón R, Hidalgo H, Viquez A & Urbina A, 1989. Atypical cutaneous leishmaniasis in a semiarid region of north-west of Costa Rica. **Transactions of the Royal Society of Tropical Medicine Hygiene 83**: 786.

# APPENDICES

**Appendix 4.1:** Abstract 430 presented during the Meeting of the American Society of Tropical Medicine and Hygiene. Cincinnati, November 1993

**Appendix 4.2:** Abstract Poster presented during the ISOPS 95. Merida, Venezuela, September 1995.

**Appendix 4.3:** Reprint of the article published in the Bulletin Entomological Research, 1996.

**Appendix 7.1:** Abstract of the talk delivered at the Royal Society of Tropical Medicine and Hygiene, 16th November 1995.

429 DEVELOPMENT OF RAPD MARKERS TO DIFFERENTIATE TWO GEOGRAPHIC RACES OF *ANOPHELES MACULATUS*, A MALARIA VECTOR IN THAILAND. Rongnoparut P*, Rattanarithikul R, and Linthicum KJ. Department of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.

The development of malaria control strategies involving genetic replacement of vector populations must include an understanding of gene flow within and among mosquito populations to be effective. *Anopheles maculatus* is a species complex that comprises important secondary vectors of malaria in southern and western Thailand. Preliminary field studies indicate that there is a hybrid zone formed between geographic races *B* and *E* of *A. maculatus* in Peninsular Thailand. This zone provides an opportunity to study gene flow between these populations of *A. maculatus*. The two races can only be identified by karyotyping the polytene chromosomes of ovarian nurse-cells, a very laborious and equivocal method. Recently, random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used for species identification and differentiation of conspecific populations. In this study we developed a method to differentiate populations of *A. maculatus*, using RAPD-PCR to establish RAPD markers. Of the 40 primers used, four primers gave unique RAPD markers for differentiating *B* and *E* race individuals. These markers are being used to identify field-collected specimens from throughout the range of each of the races and to examine the extent to which gene flow occurs.

430 HOST PREFERENCE OF *LUTZOMYIA EVANSI*, A NEW VECTOR OF VISCERAL LEISHMANIASIS IN SOUTH AMERICA. Montoya-Lerma J* and Lane RP. Fundacion Centro Internacional de Investigaciones Medicas-Cideim, Cali- Columbia; and Natural History Museum, London, UK.

Previous studies have shown the phelbotomine sand fly *Lutzomyia evansi* to be the probable vector of *Leishmania chagasi* in some foci of American visceral leishmaniasis (AVL) in Colombia and Venezuela. A longitudinal survey from January-November 1993 on the host preference selection by *Lu. evansi* was conducted in the AVL focus of San Andres de Sotavento, Cordoba, Columbia. Host preference was determined by presenting sand flies with a choice of three baits (human, dog, opossum) and a blank control in specially designed cone traps. A rotational experimental design involved 40 trapnights in which the effect of bait, proximity to forest and season could be distinguished from the potentially confounding factors of site and day to day variation. A total of seven species of phlebotomine sand fly caught in the area - *Lu. evansi, Lu. cayennensis, Lu. panamensis, Lu. gomezi, Lu. trinidadensis, Lu. rangeliana* and *Lu. shannoni*. From a total of 531 specimens of phlebotomine sand fly caught during all experiments, females and males of *Lu. evansi* accounted for 96% of the captures in the baited traps. All hosts attracted significantly more females and males of *Lu. evansi* than the control ($X^2$=14.85; df=1 p<0.001) during the sampling period. Only 15 males and two unengorged females were trapped in unbaited traps. Overall the human attracted the greatest number of flies, followed by the other two baits. Analysis of both dependent variables (attraction and feeding) showed significant differences (<0.0001; p=0.25) in relation to baits, and that sand flies moved between forest and open (=peridomicile) areas according to season. The results are discussed in terms of the causes of blood-feeding variations and their potential effects on the AVL cycle. Additionally, a comparison is made between results obtained and those from an ELISA analysis of wild blood-fed *Lu. evansi* .

431 LONGITUDINAL STUDIES OF *LUTZOMVIA EVANSI* IN A VISCERAL LEISHMANIASIS FOCUS OF COLUMBIA. Travi BL*, Gallego J, Montoya J, Jaramillo C, Llane R, and Velez ID. Fundacion CIDEIM, Cali, Columbia; and Laboratorio de Leishmaniosis, Universidad de Antioquia, Medellin, Cali, Columbia.

# SPATIAL DISTRIBUTION AND HOST PREFERENCE OF *Lutzomyia evansi* AROUND HOUSES IN COLOMBIA.

**James Montoya-Lerma & Richard P. Lane[1].**
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**[1]The Natural History Museum SW7 5BD London- UK**

*Lutzomyia evansi* has been found naturally infected with *Leishmania chagasi* in a focus of American visceral leishmaniasis (AVL) in Colombia. This sandfly is the probable vector of the disease in some areas of Venezuela. The spatial pattern of distribution and the host preferences of both host seeking and resting populations of *Lu. evansi* in an AVL focus in the Caribbean coast of Colombia, were studied by field experimentation.

A combination of sampling methods using sticky traps and direct searching was employed to determine the movement and resting patterns of wild flies indoors and along four radial transects outdoors. Flies were sampled between April (concomitant with the start of the sandfly season) and June. Frequencies of indoor catches were always less numerous than outdoor ones. Host seeking and feeding activities were detected in both sites. The resting behaviour was restricted to outdoors, although gravid females were found in indoor catches. Resting flies (including blood-fed) exhibited a patchy distribution which might be explained in terms of existence of micro-habitats in the area. Rainfall and the subsequent grade of micro-humidity also determined in great extent the degree of this patchiness. Analysis of bloodfed females by ELISA showed differences in sandfly host preference: cow being preferred (> 60 %) over human (12 %) and dog (~12 %).

Rather than confirm that *Lu. evansi* is an opportunistic feeder the results suggest that this species present endophagic and exophagic behaviour and extradomiciliar egg maturation. The importance of these events are stressed and discussed in the light of the transmission of AVL in the Colombian focus and the potential implications in areas where this sandfly is present.

LONDON

# Factors affecting host preference of *Lutzomyia evansi* (Diptera: Psychodidae), a vector of visceral leishmaniasis in Colombia

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## Abstract

Previous studies have shown the sandfly *Lutzomyia evansi* to be the vector of *Leishmania chagasi* in northern Colombia. A longitudinal survey during seven months on the host preference of *Lutzomyia evansi* was conducted in the visceral leishmaniasis focus of San Andrés de Sotavento, Colombia. Host preference was determined by presenting sandflies with a choice of three baits (human, and the reservoirs dog and opossum) and a blank control in specially designed cone traps. A rotational experimental design involved 56 trap nights in which the effect of bait, proximity to forest and season could be distinguished from the potentially confounding factors of site and day to day variation. From a total of 598 sandflies caught during all experiments, females and males of *Lutzomyia evansi* accounted for 93.8% of the captures in the baited traps. Overall, human attracted the greatest number of sand flies, followed by the other two baits. Attraction and feeding success showed significant differences ($P < 0.0001$) between baits.

## Introduction

Transmission cycles of arthropod-borne diseases are regulated by both extrinsic and intrinsic factors. Whether or not a vector will come in contact with suitable vertebrate reservoir/hosts of parasites is an important extrinsic factor (World Health Organization, 1972), while a vector's predilection for a host is an intrinsic factor. Both affect the vectorial competence of a vector, i.e. the overall ability of a vector species in a given location at specific time to transmit a pathogen.

Studies on host attraction and feeding behaviour in sandflies indicate that both New and Old World species display varying degrees of host preference, but in general all are opportunistic feeders. For instance, *Phlebotomus papatasi* (Scopoli) is considered a highly anthropophilic species by several workers in India (Dhanda & Gill, 1982; Mukhopadhyay *et al.*, 1987; Namita *et al.*, 1991) but it has been also observed biting several other animal species, in the same country (Dhanda & Gill, 1982; Namita *et al.*, 1991) and Egypt (El Sawaf *et al.*, 1989). In Sri Lanka, *P. argentipes* Annandale & Brunetti, although predominantly zoophilic in lowland areas, prefers human beings in the highlands (Lane *et al.*, 1990). Other studies reveal that the blood feeding preferences of some Ethiopian and Kenyan sandfly species are affected, among other factors, by host availability and size (Foster *et al.*, 1972; Mutinga *et al.*, 1986; Johnson *et al.*, 1993). Studies on attractiveness of sylvatic and domestic animals to *Lutzomyia* species in the New World (including the main vectors of leishmaniases) have shown that most of them are attracted by a wide number of hosts and feed upon them in an opportunistic way (Tesh *et al.*, 1971, 1972; Christensen & Herrer, 1973, 1980; Quinnell *et al.*, 1992; Morrison *et al.*, 1993). By contrast, very few species

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have distinct host preference patterns (e.g. small rodents in *L. flaviscutellata* (Mangabeira) (Shaw & Lainson, 1968), bats in *L. vespertilionis* (Fairchild & Hertig) and lizards in *L. trinidadensis* (Newstead) (Young, 1979)). The eclectic behaviour of sandfly species is a predominant and important event in zoonotic leishmaniasis cycles. The intensity of transmission of *Leishmania* parasites is determined, not only by the total number of bites to humans, but primarily by the probability of vector feeding on the vertebrate host population, which usually represents its major source of bloodmeals (Bray, 1982). Furthermore, the reservoir keeps a high infection rate of parasites (Dye, 1994).

Throughout Central and South America, *Lutzomyia longipalpis* Lutz & Neiva has been recognized as the only known vector of *Leishmania chagasi* Cunha & Chagas (Grimaldi *et al.*, 1987; Young & Arias, 1991; Grimaldi & Tesh, 1993). Remarkably, recent studies recorded *Lutzomyia evansi* Nunez-Tovar as a new vector of *Leishmania chagasi* in San Andrés de Sotavento, a focus of visceral leishmaniasis (VL) in northern Colombia, (Travi *et al.*, 1990). More recently, two *L. chagasi* flagellates have been isolated from nine other naturally infected specimens of this sandfly species (Travi *et al.*, in press). An extensive search for reservoir hosts in the area has found both dogs and the opossum *Didelphis marsupialis* Linnaeus, infected with *Leishmania chagasi* (Travi *et al.*, 1994). Thus the available evidence suggests that *Lutzomyia evansi* is the vector of *Leishmania chagasi* in San Andrés de Sotavento and dogs and *D. marsupialis* are the zoonotic reservoirs of the parasite.

Throughout its geographic range *Lutzomyia evansi* is assumed to be largely an anthropophilic species (Young & Duncan, 1994) but few workers have actually demonstrated this. Furthermore, very often the assumptions of anthropophilic behaviour are based on anecdotal data rather than on controlled experiments (c.f. Zeledón *et al.*, 1984; Feliciangeli *et al.*, 1992). In Colombia, Travi *et al.* (in press) found that *L. evansi* is the main sandfly species collected on human bait in both intra and extradomiciliary areas in the San Andrés VL focus. They also noted that, using Malaise traps, pigs attracted relatively more *L. evansi* than donkeys did. In addition, it was observed that *L. evansi* fed on a wide variety of either anaesthetized or restrained baits (dogs, ducks, chickens, rats and opossums). However, because of limitations on their experimental design they were unable to draw any conclusions on host preference. The anthropophilic behaviour of *L. evansi*, therefore, still remains unclear. The present study is the first stage in a series of experiments to determine precisely the anthropophilic behaviour of *L. evansi*.

## Methods

### *Study area*

This study was carried out in the village of El Contento, an Indian reservation occupied by approximately 300 members of the Xinú tribe, situated within the boundaries of San Andrés de Sotavento, some 110 km from the provincial capital of Montería in Córdoba, northern Colombia. The area lies at an elevation of 100 m above sea level and has a mean yearly temperature of 28°C. There are two short rainy seasons, the first in March and April and the second in August and September, although the timing and volume of precipitation can vary considerably. Typical vegetation consists of secondary dry-forest, considerably disturbed by human activity; there is no continuous forest canopy but scattered trees are present (fig. 1). Plantain, cassava and maize plantations are situated 5–200 m from houses. Dwellings are wood-framed with mud-plastered walls and palm-thatched roofs. Most have porches in which the inhabitants sit at sunset and during the early evening. The domestic animals include dogs, chickens, turkeys, pigs, cattle and donkeys. The black rat, *Rattus rattus* (Linnaeus) is abundant around human dwellings but native wild mammals are scarcely seen. A two-week survey during the study period, showed that the murid *Zygodontomys brevicauda* (Allen & Chapman) and the opossum *Didelphis marsupialis* were the predominant sylvatic species, with the rodents *Proechimys canicollis* Allen, *Heteromys anomalus* (Thompson)

Fig. 1. Typical aspect of the vegetation and housing in the visceral leishmaniasis focus in San Andrés de Sotavento, Colombia.

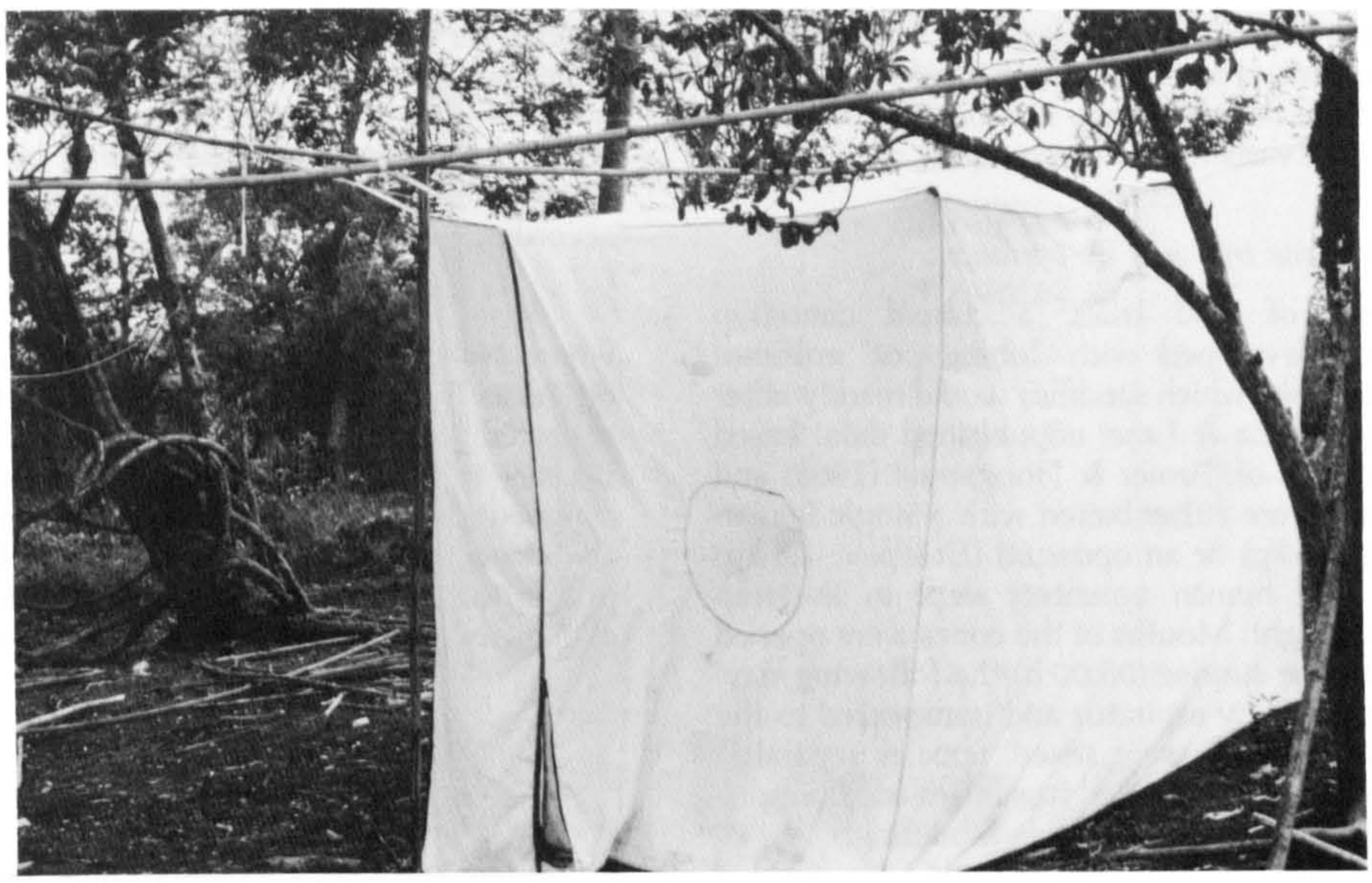

Fig. 2. A cone trap set up in the field area.

and *Oryzomys concolor* (Wagner) also present in the area (G. Adler, pers. comm.). The area is endemic for both VL due to *Leishmania chagasi* and cutaneous leishmaniasis due to *L. panamensis* (Travi *et al.*, 1990).

### *Experimental design*

Host preference was determined by presenting a choice of three baits (human, dog, opossum) and a blank control in specially designed cone traps (fig. 2) as part of a rotational experimental design. Figure 3 indicates the arrangement of the four traps. Each trapping position was 5 m from the wall of a house. Each host, together with its trap (to avoid bias from residual odour), was rotated to the next trapping position the following night. Thus, after four consecutive nights each bait or the control had been presented at each trapping position to give four replicates.

To determine the effect of proximity to the 'forest', this four-nightly procedure was carried out around two houses in the village, one house with the forest edge within 5 metres, the other house with the forest edge more than 10 metres away, termed forest and 'open area', respectively. The houses were matched for the domestic animal composition and the human:domestic animal ratio. These factors remained relatively constant throughout the study period. To detect any seasonal change in host preference, the experiment (eight trap-nights) was repeated seven times throughout the year to cover periods in the rainy season (March–April and August–September) as well as the drier seasons (June–July and October–November).

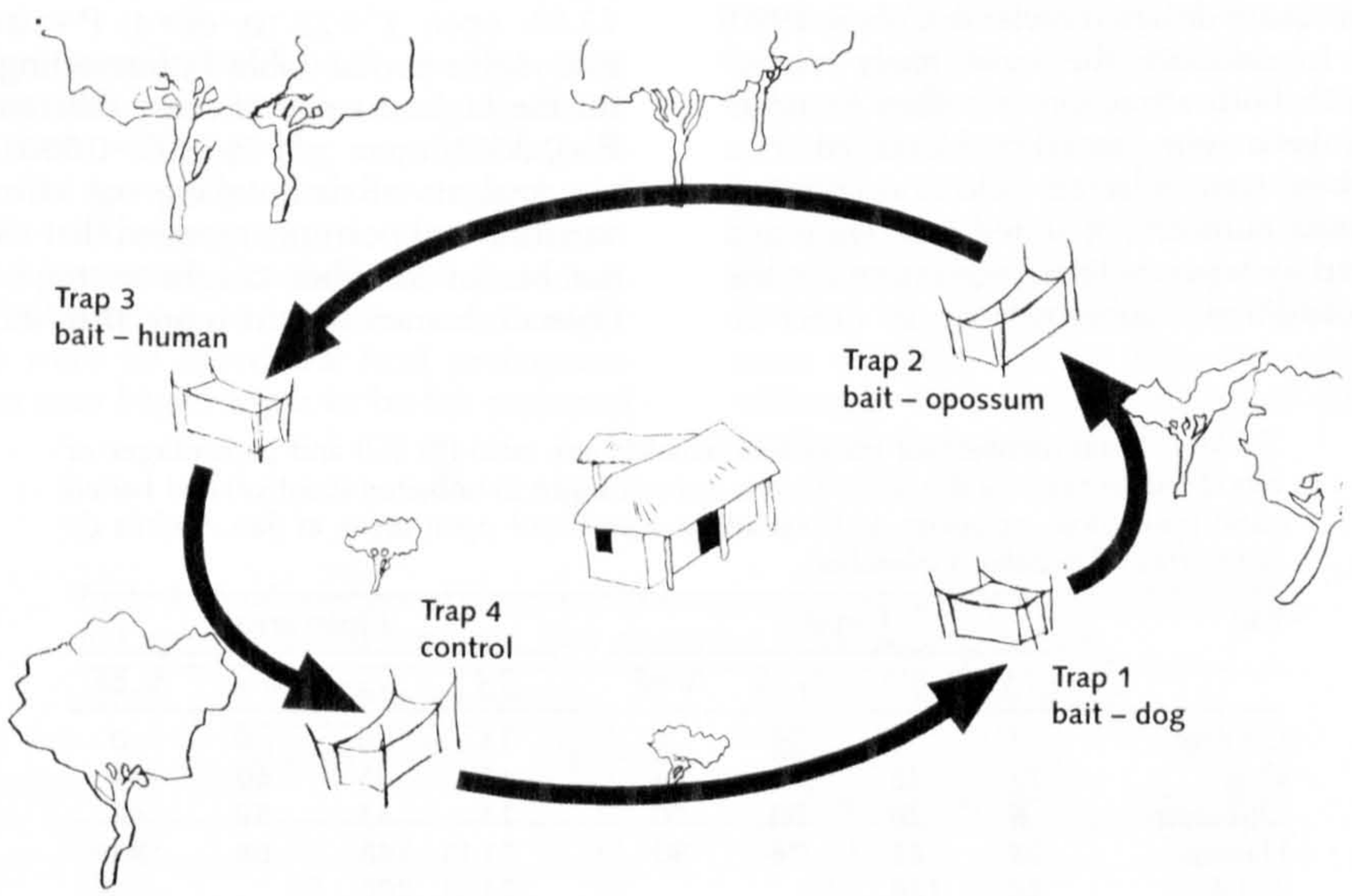


Fig. 3. Arrangement of cone traps and their baits; each was rotated daily as indicated by arrows.

Thus the total experiment involved 56 trap-nights in which the effect of bait, proximity to forest and season could be distinguished from the potentially confounding factors of site and day to day variation.

### *The cone trap and its operation*

After a series of field trials, a cuboid cone-trap (2×1.5×2 m) was developed with 'lobster-pot' entrance cones facing inward into which sandflies would readily enter (fig. 2 & Montoya-Lerma & Lane, unpublished data) based on earlier trap designs of Turner & Hoogstraal (1965) and Ward (1977). Traps were either baited with a single human (ca. 70 kg), a dog (20 kg) or an opossum (*Didelphis*, 2.5 kg) in a wide cage. The human volunteer slept in the trap without the use of a light. Mouths of the cones were opened from 19.00 h to before sunrise (06.00 h) the following day. Sandflies were removed by aspirator and transported to the field laboratory where they were sexed, females separated into fed and unfed, and all identified to species according to Young (1979) after dissection and slide-mounting.

### *Sticky trap sampling*

Simultaneously with the operation of the cone-traps, sticky traps (sheets of paper A4 size, coated in castor oil) were set overnight in and around houses so that the species composition could be compared with that in the cone traps. On one occasion (July), the species composition on domestic animals (a tethered cow and pig) was measured by aspirating sandflies for five minutes each hour throughout three peak hours of sandfly activity.

### *Data analysis*

As mentioned above, the focus of this paper is to compare the relative attractiveness of baits in different habitats. During a pilot study (data not shown) it was found that the distribution of sandflies between nights and between the different baits, was not normal but approximated a Poisson distribution. A Kruskal-Wallis analysis of variance (ANOVA) for a Latin square design (Fowler & Cohen, 1990) was therefore used to calculate the most likely marker (factor) associated with both attraction (sandflies found in a trap) and feeding behaviour (sandflies blood-fed in a trap). Once the markers were selected (selection criterion: $P < 0.25$) the expected numbers of unfed and engorged females were estimated by separate linear regression models assuming unbaited conditions. Subsequently, in order to control for other factors (season, forest, night, position), the expected values were used as covariables in a covariance analysis with one factor (bait). A chi-squared analysis was used to compare the blood-feeding between baits and between habitats. Finally, the feeding index (FI) was calculated according to Kay *et al.* (1979):

$$FI=(Ne/NE')/(Ef/Ef')$$

where Ne and Ne' are the number of sandfly blood feeds on hosts 1 and 2, respectively, and Ef and Ef' are the expected proportion of feeds on hosts 1 and 2, respectively. Because this experiment tests attraction to a bait, we have assumed that entering a trap is independent of the local abundance of the host animal in calculating the expected proportions. Thus a FI greater than 1 indicates an increased amount of feeding on host 1 relative to host 2.

## Results

### *Species composition*

Seven species of phlebotomine sandfly were caught in the traps – *Lutzomyia evansi*, *L. cayennensis* (Floch & Abonnenc), *L. panamensis* (Fairchild & Hertig), *L. gomezi* (Nitzulescu), *L. trinidadensis* (Newstead), *L. rangeliana* (Ortiz) and *L. shannoni* (Dyar). Of these, *L. evansi* was the most numerous in each baited-trap. Other species were captured less frequently. There was no significant difference in species composition between cone traps and sticky traps, although there were differences in the species proportions and in seasonal patterns.

### *Host attractiveness*

From a total of 598 specimens of phlebotomine sandfly caught in cone traps during the complete experiment, 83.8% were *L. evansi* (females and males). All data analysis focused on *L. evansi* because of its abundance and epidemiological importance. Only 14 males and three unfed females were caught in unbaited traps. In both habitats all hosts attracted significantly more *L. evansi* than the controls (forest $\chi^2=23.50$; open $\chi^2=25.10$; df=4; $P < 0.0001$) during the 56 trap-nights period (table 1). Interestingly, females accounted for the highest value of these differences (forest, $\chi^2=19/9$; $P=0.0002$; open $\chi^2=36.6$; $P=0.0001$; df=3).

Analysis of data, taking out effects of night to night variation and position, revealed that there was a trend in the number of sandflies caught in traps with different baits. Overall, human caught more than either dog or opossum

Table 1. Total numbers of males and females; sex ratio (% ♀♀) and percentages of blood-fed females (% BF) of *Lutzomyia evansi* caught in unbaited (control) and baited cone traps (dog, opossum and human) in forest and open areas in San Andrés de Sovavento (Córdoba, Colombia).

| Bait | Forest | | | | Open area | | | |
|---|---|---|---|---|---|---|---|---|
| | ♂♂ | ♀♀ | % ♀♀ | % BF | ♂♂ | ♀♀ | % ♀♀ | % BF |
| Control | 1 | 3 | 75 | 0 | 13 | 0 | 0 | 0 |
| Dog | 11 | 25 | 70 | 84 | 17 | 25 | 60 | 64 |
| Opossum | 5 | 20 | 80 | 70 | 23 | 33 | 59 | 49 |
| Human | 25 | 81 | 78 | 90 | 71 | 148 | 68 | 33 |
| Total | 42 | 129 | | | 124 | 206 | | |
| Mean | | | 76 | 81 | | | 47 | 49 |

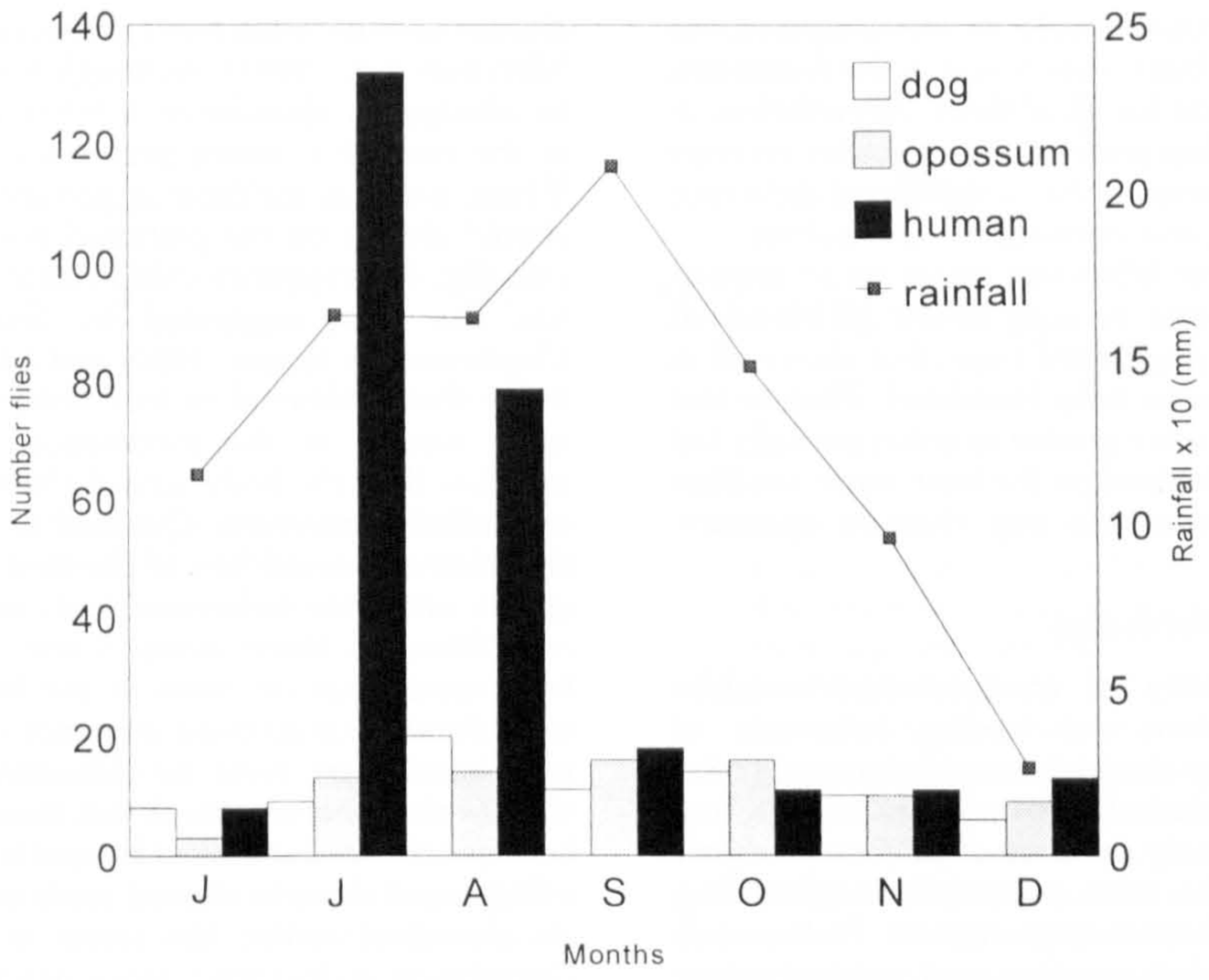


Fig. 4. Number of *Lutzomyia evansi* caught monthly in baited traps between June and December 1992, in San Andrés de Sotavento, Colombia.

(table 1, $P < 0.05$) but there was no significant difference between dogs and opossum ($P > 0.05$). This host preference is the same in the forest and open areas. Traps in the open habitat (table 1) caught more sandflies than in the forest habitat (for all hosts), but this difference was not statistically significant (0.1062; $P = 0.25$).

*Seasonal host-preferences*

Total sandfly captures numbers from forest and open areas were pooled to detect any seasonal changes in the host preference of *L. evansi*. Firstly, there was a clear seasonal variation in sandfly abundance (fig. 4), with the majority caught in July–August.

At the end of April, concomitantly with the first rains, sandfly numbers were low and there was no significant difference between baits. During the month of peak abundance, humans were greatly preferred over dogs and opossums. In August, catches began to decrease but man was still the preferred host until October when there was no clear preference, but a few more sandflies were caught on opossums. There were no detectable host preferences until December, when man began again to be the preferred host.

*Sex ratio*

In all baited-traps, the sex ratios were always biased in favour of females (table 1). However, in control traps, males were relatively more abundant, and more so in the open area (38%) than in the forest (25%). There was no significant difference in sex ratios between hosts, though in all cases, the absolute number of females was higher on human than on other baits.

*Feeding behaviour*

From a total of 332 host-seeking feamles caught in the baited cone-traps only 57% of them were blood-fed, but the proportion blood-fed showed significant differences between baits ($F = 13.27$; $df = 4$; $P < 0.0001$). A $\chi^2$ analysis of blood-feeding between pairs of baits showed that in the forest site more *L. evansi* females fed on man than they did on opossum ($\chi^2 = 5.44$ $P < 0.05$) but no significant differences were found between man and dog ($\chi^2 = 0.71$, $P > 0.05$) or between dog and opossum. In contrast, in the open area, more sandflies fed on dog and opossum than on man, (although there was no statistical difference between the levels on man and opossum).

Table 2. Total numbers of blood-fed *Lutzomyia evansi* females caught in human, dog and opossum-baited traps and their respective blood feeding indices.

| Area | Blood-fed females | | | Feeding index | | |
|---|---|---|---|---|---|---|
| | Human | Dog | Opossum | Human: dog | Human: opossum | Dog: opossum |
| Forest | 73 | 21 | 14 | 1.07 | 1.30 | 1.20 |
| Open | 49 | 16 | 16 | 0.52 | 0.68 | 1.32 |
| Total | 122 | 37 | 30 | – | – | – |

When the feeding indices (table 2) are compared, no clear differences between baits were found in the forest site. Feeding indices were similar for all of them. Nevertheless, in the open area sandfly feeding preference was greater on dogs and opossums than on humans. Also, a significant difference was found between dog and opossum in this habitat.

We suspected that host behaviour might be an important factor in feeding success because almost all blood-fed females from human and dog-baited traps (but above all in the former) were found to be fully blood-fed. Thus to test this, all blood-fed females were graded as either partially-fed or fully-fed. When they did feed on the host, more sandflies were more fully-fed on man and dog than on opossum.

## Discussion

There are a multiplicity of environmental variables affecting the host selection and feeding behaviour of sandflies, including the size, abundance and behaviour of the hosts, the habitat, and abiotic factors such as temperature and wind. In the present study, basic assumptions and efforts to control and reduce the main potentially confounding factors were made throughout the experiments. Factors such as trapping site, day to day variation and residual odour between hosts were eliminated by daily rotation of the baits together with their traps (including plastic ground-sheet). Potential biases due to abundance of the hosts were controlled by holding domestic animals remote from the baited traps. We assumed that all trapped sandflies were actually host-seeking individuals since the differences between the control and other traps was so great. The presence of blood-fed females in baited traps, confirms the female's attraction to a specific host.

The dominant species in the visceral leishmaniasis focus of San Andrés de Sotavento is *L. evansi*, with other species poorly represented in all our experiments. Early studies (Travi *et al.*, in press) in the same area demonstrated a similar pattern with *L. evansi* constituting 92% and 97.3% of the total catch on human bait (n=909) and in Shannon traps (n=6697), respectively.

The bimodal seasonal distribution in sandfly abundance measured by the newly developed cone-trap generally follows that of Travi *et al.* (in press) using a number of techniques. This indicates that the cone-trap does not introduce any systematic bias into the sampling. The highest number of sandflies were caught at the beginning of the rainy season followed by variable, but sustained, activity of the flies during the rainy season before the numbers fell away in the dry season. Attempts to conduct experiments during the summer period (February–March) were unsuccessful because the sandflies were so scarce.

The results show quite clearly that overall there is a clear preference for humans over either dogs or opossums ($P < 0.05$), and that there is no measurable difference between dogs and opossums (table 2). Curiously, these preferences are not equally distinct over all sandfly densities (fig. 4); at low levels of sandfly abundance there is no statistically significant preference for a host but when the sandfly abundance is high (July and August), there is a marked preference for humans. These results indicate that at low densities the sandflies are opportunistic feeders.

It has been postulated that attraction to humans by some visceral leishmaniasis vectors (e.g. *L. longipalpis*) is a function of the size and number of hosts in the area rather than an intrinsic attraction to humans (cf. Quinell *et al.*, 1992; Morrison *et al.*, 1993). Although host preferences may well be affected by abundance of hosts, our results suggest that in the case of *L. evansi* preference is independent of size. If host size was the most important factor then human bait should always be the preferred host, but this was not the case (fig. 4). Preference independent of host size in sandflies has also been suggested by Shaw & Lainson (1968), Christensen & Herrer (1980) and Mutinga *et al.* (1986). It is likely that additional factors (intrinsic and extrinsic) have more weight in the modulation of host preference in sandflies than the body size of the host. Recently, in a well controlled experiment, Quinnell & Dye (1994) found that the relative accessibility of the host is more crucial than its size in attraction behaviour in *L. longipalpis*.

Although there were some interesting differences between catches on hosts in the forest and open habitats (e.g. blood feeding) these were not consistent or systematic in nature. There were no differences in host preferences. One explanation for this is that there is no clear distinction between the habitat around houses in forested sections of the villages and those in cleared areas in the middle of villages. As described earlier, the forest in this part of Cordoba Department is dry forest, more akin to scrub than the dense, high and wet forest elsewhere in Colombia (Bajo Calima or Tumaco (Travi *et al.*, 1988)), where such a difference between village centre and periphery might be more overt.

In general, sandflies were more abundant in the open habitat, contrary to our expectations, and this was most apparent during the months of July, August, September and December. We conclude from this result that *L. evansi* is well adapted to disturbed habitats around human dwellings and that any attempt to control this species by clear felling of forest (Esterre *et al.*, 1986) may not be successful.

The sex ratio of the catches was dominated by females (table 1) suggesting that *L. evansi* does not form large aggregations of males on hosts as seen in *L. longipalpis* and *Phlebotomus argentipes* (Lane *et al.*, 1990), assuming that the trap design did not interfere with male location of hosts. The lack of males in the traps precludes attraction of females simply in response to male aggregations.

The number of blood-fed sandflies found in traps varied between hosts and habitats. There was a distinct hierarchy in the number of blood-fed sandflies found in the traps: more fed fully on humans than on dogs and more on dogs than on opossums. This feeding was independent of habitat. These results have significant epidemiological implications. According to Bray (1982) an ideal reservoir for *Leishmania* spp. should not only be attractive to a sandfly vector (measured in our case by the total number of sandflies caught in the traps of each bait) but also be the major source of bloodmeals for the reservoir. However, in San Andrés de Sotavento although opposums and dogs display the highest rates of *Leishmania chagasi* infection (Travi *et al.*, 1994) our results suggest they do not represent the main source of blood for *Lutzomyia evansi* (total number of blood-fed flies on opossums is less than the two other hosts tested). This apparent paradox can also be deduced from the data of Morrison *et al.* (1993) working in the VL focus in Melgar, Colombia and was noted by Quinnell *et al.* (1992) in Brazil who found low attraction to dogs but a high level of infection. Part of the explanation for these observations can be gleaned from closer examination of the amount of blood consumed by the fed sandflies in the traps.

Table 3. Comparisons between the number of blood-fed *Lutzomyia evansi* caught in baited traps in forest and open habitats in San Andrés de Sotavento, Colombia, and their feeding success.

| Host | Number of blood-fed flies | | | | Feeding success | | | |
|---|---|---|---|---|---|---|---|---|
| | Forest | | Open | | Forest | | Open | |
| | $\chi^2$ | $P$ | $\chi^2$ | $P$ | $\chi^2$ | $P$ | $\chi^2$ | $P$ |
| Human–dog | 0.71 | 0.40 | 8.20 | 0.004 | 1.68 | 0.20 | 8.59 | 0.003 |
| Human–opossum | 5.41 | 0.019 | 2.50 | 0.12 | 17.44 | 0.000 | 0.23 | 0.63 |
| Dog–opossum | 1.36 | 0.26 | 1.48 | 0.24 | 5.17 | 0.22 | 4.40 | 0.035 |

When sandflies did manage to feed on a host, more were fully fed on humans and dogs than on opossums (table 3). We interpret these results in terms of the defensive ability of the hosts. Opossums are more nocturnally active than humans or dogs and therefore during the night time, when the experiments were conducted, were in a better position to defend themselves against sandfly feeding. Wild caught *L. evansi* females fed more avidly on restrained or anaesthetized opossums than they did on wild rodents, dogs and unrestrained opossums (Travi & Montoya, unpublished data), a similar phenomenon was found in mosquitoes by Day & Edman, (1984).

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## References

**Bray, R.S.** (1982) The zoonotic potential of reservoirs of leishmaniasis in the Old World. *Ecology of Disease* **1**, 257–267.

**Christensen, J.A. & Herrer, A.** (1973) Attractiveness of sentinel animals to vectors of leishmaniasis in Panama. *American Journal of Tropical Medicine and Hygiene* **22**, 578–584.

**Christensen, H.A. & Herrer, A.** (1980) Panamanian *Lutzomyia* (Diptera: Psychodidae). Host attraction profiles. *Journal of Medical Entomology* **17**, 522–528.

**Day, J.F. & Edman, J.D.** (1984) Mosquito engorgement on normally defensive host depends on host activity patterns. *Journal of Medical Entomology* **21**, 732–740.

**Dhanda, V. & Gill, G.S.** (1982) Double blood meals by *Phlebotomus argentipes* and *Ph. papatasi* in two villages of Maharashtra. *Indian Journal of Medical Research* **76**, 840–842.

**Dye, C.** (1994) Vector control. pp. 159–174 *in* Scott, M.E. & Smith, G. (*Eds*) *Parasitic and infectious diseases*. London, Academic Press.

**Esterre, P.** (1986) Evaluation d'un programme de lutte contre la leishmaniose cutanée dans un village forestier du Guyane française. *Bulletin of the World Health Organization* **64**, 559–565.

**El Sawaf, B.M., Mansour, N.S., El Said, S.M., Daba, S., Yoursef, F.G., Kenawy, M.A. & Beier, J.C.** (1989) Feeding patterns of *Phlebotomus papatasi* and *Phlebotomus langeroni* (Diptera: Psychodidae) in El Agamy, Egypt. *Journal of Medical Entomology* **26**, 497–498.

**Feliciageli, M.D., Arredondo, C. & Ward, R.** (1992) Phlebotomine sandflies in Venezuela: review of the *verrucarum* species group (in part) of *Lutzomyia* (Diptera: Psychodidae) with description of a new species from Lara. *Journal of Medical Entomology* **29**, 729–744.

**Foster, W.A., Boreham, P.F.L. & Tempelis, C.H.** (1972) Studies on leishmaniasis in Ethiopia. IV: feeding behaviour of *Phlebotomus longipes* (Diptera: Psychodidae). *Annals of Tropical Medicine and Parasitology* **66**, 433–443.

**Fowler, J. & Cohen, L.** (1990) *Practical statistics for field biology*. 227 pp. Milton Keynes, Open University Press.

**Grimaldi, G. & Tesh, R.B.** (1993) Leishmaniases of the New World: Current concepts and implications for future research. *Clinical Microbiology Reviews* **6**, 230–250.

**Grimaldi, G. Jr, David, J.R. & McMahon-Pratt, D.** (1987) Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* **36**, 270–287.

**Johnson, R.N., Ngumbi, P.M., Mwanyumba J.P. & Roberts, C.R.** (1993) Host feeding preference of *Phlebotomus guggisbergi*, a vector of *Leishmania tropica* in Kenya. *Medical and Veterinary Entomology* **7**, 216–218.

**Kay, B.H., Boreham, P.F.L. & Williams, G.M.** (1979) Host preferences and feeding patterns of mosquitoes (Diptera: Culicidae) at Kowanyama, Cape York Peninsula, northern Queensland. *Bulletin of Entomological Research* **69**, 441–457.

**Lane, R.P., Pile, M.M. & Amerasinghe, F.P.** (1990) Anthropophagy and aggregation behaviour of the sandfly *Phlebotomus argentipes* in Sri Lanka. *Medical and Veterinary Entomology* **4**, 79–88.

**Morrison, A.C., Ferro, C. & Tesh, R.B.** (1993) Host preferences of the sandfly *Lutzomyia longipalpis* at an endemic focus of American visceral leishmaniasis in Colombia. *American Journal of Tropical Medicine and Hygiene* **49**, 68–75.

**Mukhopadhyay, A.K., Kumar, K. & Rahman, S.J.** (1987) Host preference of phlebotomine sandflies in and around Delhi. *Journal of Communicable Diseases* **19**, 408–411.

**Mutinga, M.J., Kyai, F.M., Kamau, C. & Omogo, D.M.** (1986) Epidemiology of leishmaniasis in Kenya. III. Host preference studies using various types of animal baits at animal burrows in Marigat, Baringo District. *Insect Science and its Application* **7**, 191–197.

**Namita, M., Joshi, V. & Bansal, S.K.** (1991) Host preference pattern of phlebotomine sandflies of Bikaner city. *Indian Journal of Medical Research* **93**, 328–329.

**Quinnell, R.J. & Dye, C.** (1994) An experimental study of peridomestic distribution of *Lutzomyia longipalpis* (Diptera: Psychodidae). *Bulletin of Entomological Research* **84**, 379–382.

**Quinnell, R.J., Dye, C. & Shaw, J.J.** (1992) Host preferences of the phlebotomine sandfly *Lutzomyia longipalpis* in Amazonian Brazil. *Medical and Veterinary Entomology* **6**, 195–200.

**Shaw, J.J. & Lainson, R.** (1968) Leishmaniasis in Brazil: II.

Observations on enzootic rodent leishmaniasis in the lower Amazon region–the feeding habits of the vector, *Lutzomyia flaviscutellata* in reference to man, rodents and other animals. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **62**, 396–405.

**Tesh, R.B., Chianiotis, B.N., Aronson, M.D. & Johnson, K.M.** (1971) Natural host preferences of Panamanian sandflies as determined by precipitin test. *American Journal of Tropical Medicine and Hygiene* **20**, 150–156.

**Tesh, R.B., Chianiotis, B.N., Carrera, B.R. & Johnson, K.M.** (1972) Further studies on the natural host preferences of Panamanian phlebotomine sandflies. *American Journal of Epidemiology* **95**, 88–93.

**Travi, B.L., Montoya, J., Solarte, Y., Lozano, L. & Jaramillo, C.** (1988) Leishmaniasis in Colombia I. Studies on the phlebotomine fauna associated with endemic foci in the Pacific coast region. *American Journal of Tropical Medicine and Hygiene* **39**, 261–266.

**Travi, B.L., Vélez, I.D., Brutus, L., Segura, I., Jaramillo, C. & Montoya, J.** (1990) *Lutzomyia evansi*, an alternate vector of *Leishmania chagasi* in a Colombian focus of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine* **84**, 676–677.

**Travi, B.L., Jaramillo, C., Montoya, J., Segura, I., Zea, A., Goncalves, A. & Velez, I.D.** (1994) *Didelphis marsupialis*, an important reservoir of *Trypanosoma* (*Schizotrypanum*) *cruzi* and *Leishmania* (*Leishmania*) *chagasi* in Colombia. *American Journal of Tropical Medicine and Hygiene* **50**, 557–565.

**Travi, B.L., Gallego, J., Montoya, J., Jaramillo, C., Llano, R. & Velez, I.D.** (in press) Bionomics of *Lutzomyia evansi* (Diptera: Psychodidae), vector of visceral leishmaniasis in northern Colombia. *Journal of Medical Entomology*.

**Turner, E.R. & Hoogstraal, H.** (1965) Leishmaniasis in the Sudan Republic. 23. Sandflies (*Phlebotomus*) attracted to rodent-baited traps. *Journal of Medical Entomology* **2**, 137–139.

**Ward, R.D.** (1977) The colonisation of *Lutzomyia flaviscutellata* (Diptera: Psychodidae), a vector of *Leishmania mexicana amazonensis* in Brazil. *Journal of Medical Entomology* **14**, 469–476.

**World Health Organisation** (1972) Vector ecology. *WHO Technical Report Series* **501**, 1–38.

**Young, D.G.** (1979) A review of the bloodsucking psychodid flies of Colombia (Diptera: Phlebotominae and Sycoracinae). *University of Florida Agricultural Experimental Stations Bulletin* (*Technical*) **806**, 1–266.

**Young, D.G. & Arias, J.R.** (1991) Phlebotomine sandflies in the Americas. *Pan-American Health Organization Technical Paper* **33**, 1–26.

**Young, D.G. & Duncan, M.A.** (1994) Guide to the identification and geographic distribution of *Lutzomyia* sandflies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). *Memoirs of the American Entomological Institute* **54**, 1–881.

**Zeledón, R., Murillo, J. & Gutiérrez, H.** (1984) Observaciones sobre la ecología de *Lutzomyia longipalpis* (Lutz & Neiva, 1912) y posibilidades de existencia de leishmaniasis visceral en Costa Rica. *Memoirs Instituto Oswaldo Cruz* **79**, 455–459.

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**Assessing recentcy of association in Lutzomyia and Leishmania**

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Throughout South America, most Leishmania parasite species are transmitted by one particular species of sandfly. However, in some localities the principal vector is absent and the parasite is transmitted by other species. A particularly good example is the transmission of Le. chagasi (causing visceral leishmaniasis) by the widespread Lu. longipalpis but occasionally by Lu. evansi. To determine if the partners in these less frequent vector-parasite combinations are well adapted, parasite development in an occasional vector was assessed by experimental and natural infections.

No significant differences were found between Lu. longipalpis (the dominant vector) and Lu. evansi (an occasional vector) in experimental infection rates but the latter species was less heavily infected, had fewer metacyclic forms and had a higher mortality. From these results, it is concluded that the Le. chagasi - Lu. evansi association is more recently evolved.